

Chernobyl's legacy to science

The consequences of the Chernobyl accident have given health physicists and geneticists a wealth of information about the effects of radiation exposure. Some is reassuring, some less so, but support for structured follow-up studies remains essential.

It might be seen as churlish to look for a silver lining in a cloud as grey and threatening as that caused by the explosion of the Unit 4 reactor at Chernobyl, exactly ten years ago tomorrow. Whatever the precise figures involved turn out to be, the human suffering involved was immense. It includes that of the tens of firemen who died after fighting the fire that followed, of the many hundreds of children who have since developed thyroid cancers from ingesting radioactive iodine, and of the hundreds of thousands of people who suffered severe psychological stress as a result of having to leave their homes and communities.

Furthermore, as last weekend's G7 summit meeting in Moscow demonstrated, the danger is not yet over. Few are confident about the safety of nuclear reactors in the former Soviet Union, and many will sleep peacefully only when the 15 Chernobyl-style reactors — including those still operating at Chernobyl itself — have been taken out of service.

It would, however, be equally churlish to deny the extent to which medical, environmental and scientific studies of the consequences of the accident in the intervening ten years have helped researchers to refine their knowledge of the impact of radiation on living organisms. This knowledge has many important implications. To take but one example, the cost-effectiveness of the massive effort being undertaken in the United States to clean up the nuclear facilities of the Department of Defense depend critically on the social acceptability of radiation levels that will be left after the clean-up has been completed. If there is a threshold below which radiation has no long-term biological effect, will much be gained by achieving complete elimination? Conversely, if no threshold exists, can the costs of eliminating radiation risks entirely be justified by the likely medical benefits if these are, ultimately, insignificantly small?

In this context, the Chernobyl accident has at the least provided researchers with a unique scientific and medical experiment. Some of the lessons were immediate; one, for example, was the relative ineffectiveness of the bone-marrow transplantation used in a desperate attempt to save some of those who had received massive exposures after the accident. Such transplants can work in a hospital setting, where closely controlled radiation can ensure the complete elimination of an individual's own bone marrow before its replacement. But where an individual's exposure has been uncontrolled and uneven, residual bone marrow, we now know from experience, quickly sets up an immune response leading to rejection of the implant.

Other lessons have, at least so far, been relatively reassuring for health physicists. The high levels of thyroid cancer among children in the region, with more than 400 such cases reported from Belarus since 1990, as well as the relative absence of an increased level among other age groups, are broadly in line with previous expectations, based on calculations of the likely doses that would have been received by members of families in which advice to take iodine prophylaxis and not to eat home-grown vegetables and locally produced milk were ignored or given too late. Conversely, the apparent absence so far of any documented leukaemia among exposed population groups is also consistent with predicted dose-response relationships based on a relatively low exposure to caesium in the ground.

Some of the potential lessons remain to be addressed. One tantalizing prospect facing cancer epidemiologists is that of carrying out mortality and morbidity surveys of the roughly 600,000 individuals who acted as so-called 'liquidators' clearing up the Chernobyl area after the accident. Both the relative consistency of the exposure received by these individuals, who were removed from the area as soon as such doses exceeded 100 millisieverts, and their genetic proximity to large potential control populations in Western Europe promise data that could be as significant in analysing the impact of radiation exposure as that gained from the Japanese atomic bomb survivors — the main data on which modern radiation standards are set, both for those working in the nuclear industry and for environmental exposure.

Finally, as two papers published in this issue of *Nature* illustrate, genetic studies of both human and animal populations, using techniques far more advanced than those available at the time of the Japanese studies, promise new insights into the precise way in which radiation interacts with living organisms — and how such organisms respond (see pages 683–686, 707–708 and 665). Some of these data are potentially disturbing; an unusually high level of length changes in nuclear minisatellite loci among individuals living even a few hundred kilometres away from the accident, providing apparent evidence of germline alterations due to low levels of radiation, merits further study despite uncertainties in mutation rates and radiation exposure. Other results are intriguing; one question raised by evidence of unexpectedly high rates of substitution in protein-coding genes in voles living near the stricken reactor is why these animals appear to be surviving successfully under high radiation levels.

Furthermore, Chernobyl could still spring some surprises, both in Russia and elsewhere. Few Western scientists are prepared to accept at face value claims made earlier this month by Ukrainian health officials that 125,000 people have already died as a result of the accident. Scepticism has also greeted other suggestions made last week that the death rate may be much higher than Western scientists are predicting (see page 658). Without the clear presentation of the relevant clinical evidence — including detailed comparison to control populations — in peer-reviewed journals, many in the West will be reluctant to accept conclusions that contradict conventional wisdom. Yet it would be foolhardy to reject all such claims on this basis; certainly the more plausible justify continued monitoring.

Much of the damage caused by the Chernobyl accident — including the severe dent in the safety reputation of the world's nuclear industry — is likely to be irreversible. Much of it has yet to emerge; one of the most upsetting facts facing those studying its long-term consequences is that the incidence of thyroid cancers among exposed children is likely to remain high for many years to come. And much remains to be learnt of the long-term physical and psychological consequences. Perhaps the most significant compensation that politicians can offer for the enormous human and environmental costs of the Chernobyl accident would be to make sufficient funds available to ensure — for example by a detailed long-term study of the Chernobyl 'liquidators' — that the unique opportunity for increasing our knowledge of the true dangers of radiation is not lost. □



Now Europe's physicists seek shift in strategy for fusion research

Paris. The International Thermonuclear Experimental Reactor (ITER) project, already reeling from a recent US decision to slash funding for fusion research, is facing a further blow — a report by a panel of physicists that calls on the European Union (EU) to rethink its strategy for fusion research.

ITER, formally launched in 1988 as a collaborative project between the EU, the United States, Russia and Japan, aims to demonstrate the feasibility of controlled nuclear fusion by building a huge tokamak reactor with three main goals: igniting a magnetically confined deuterium-tritium plasma; sustaining fusion for 1,000 seconds; producing 1,500 MW of thermal power.

The project is in the engineering design phase, with the ITER partners scheduled to decide in 1998 whether to build the reactor and, if so, where. Prospects that it will be built improved this month with reports that Japan may be prepared to pay for up to 70 per cent of the total costs—estimated at more than \$10 billion — provided that the machine is constructed there (see below).

Nonetheless, the wisdom of proceeding with ITER as envisaged is increasingly being questioned, particularly in the United States (see *Nature* 375, 713; 1995). Cracks in support for ITER are now also appearing in Europe, previously a stalwart supporter of magnetic confinement fusion (MCF), and home to the world's most advanced tokamak, the Joint European Torus (JET) in the United Kingdom.

A report commissioned by the European

Science and Technology Assembly (ESTA), an official advisory body to the European Commission in Brussels—a draft of which has been seen by *Nature* — asserts that, while some of the obstacles to ITER are political and economic, “there is increasing awareness of *physical-technical uncertainties* in the project that suggest that more scientific knowledge is needed”.

The main thrust of the report, produced by an ESTA working party on “Inertial Confinement Options to Controlled Nuclear Fusion”, is that the EU is making the mistake of putting all its eggs in one basket by spending almost all its annual funding of ECU200 million (US\$250 million) for fusion on magnetic confinement fusion. In particular, the report argues that Europe is neglecting the potential of an alternative approach to fusion, namely inertial confinement fusion (ICF).

Fusion is obtained by forcing light nuclei to collide at high velocities in a plasma at temperatures of millions of degrees. Because all materials vapourize at such temperatures, the plasma must be confined without contact. MCF devices, such as tokamaks, do this using magnetic fields. In contrast, ICF uses powerful lasers or heavy-ion beams to compress a pellet of fuel—such as deuterium and tritium—and heat it to 50 million degrees, triggering thermonuclear fusion that increases the temperature further to 400 million degrees.

Confinement is obtained by using a pellet



Taking aim: laser research could benefit from a shift of funds to inertial confinement fusion.

so small that the fusion reaction burns out before the plasma expands significantly. In practice, a few milligrams of fuel would be ignited for less than a nanosecond. A power-generating inertial confinement reactor that ignited one such pellet per second could produce 1,000 MW of thermal energy.

Research into the use of inertial confinement to generate power has been restricted until recently because much of it has been classified, as its main use has been to simulate the explosion of hydrogen bombs (see *Nature* 380, 8; 1996). To this end, the United States is building the \$1.8-million National Ignition Facility (NIF) at the Lawrence Livermore Laboratory in California, while France plans a similar facility, the FF6-billion Megajoule laser in Bordeaux.

The United States declassified much inertial confinement research in 1993, however. The picture that has subsequently emerged of the status of such research shows that it is a “serious alternative candidate” to magnetic confinement fusion for power generation, according to the ESTA report. Europe’s exclusive focus on MCF, it concludes, is therefore “unbalanced and no longer justified” (in Japan, in contrast, ICF is already being actively pursued).

To redress the balance, the report calls on the EU to establish immediately a modest programme in inertial confinement research, with initial financial support running at around 10 per cent of the total fusion budget. The report adds that, while such a programme would cover basic research on inertial confinement, it would “have a power producing inertial confinement reactor as its ultimate goal”.

The European Science Foundation has agreed to draft a detailed proposal to assess both existing resources in Europe and possibilities for access to facilities such as the NIF, the planned French Megajoule laser and the Sprite excimer laser at the UK Rutherford Appleton Laboratory. ►

Japan's bid could be the making of ITER

Paris. “I’m still confident that ITER or something similar will be built,” says Martin Keilhacker, director of the Joint European Torus (JET). One reason for his confidence is that Japan may be willing to remove the major obstacle to the construction of ITER — money.

Earlier this month, Japan made an informal proposal to have ITER built there at a meeting of the working group responsible for studying funding of the reactor. Under the proposal, 40 per cent of the costs of ITER — corresponding to the cost of the core — would be shared equally among the four international partners. But Japan would shoulder the rest of the costs, leaving it with 70 per cent of the total costs.

The Japanese offer is said to have

been welcomed by the United States, as it accords with US reluctance to pay more than 10 per cent of the costs of ITER. If the offer is made formally it would end the prospect of Europe hosting the machine. “it is inconceivable that Europe could match this offers,” says Keilhacker. “But it’s an offer that would be difficult to say no to.”

Although the Japanese offer is being taken seriously by fusion researchers, some argue that financing at such a high level would meet with resistance from within the Japanese fusion community, as it would inevitably reduce funding for domestic fusion programmes. If the Japanese offer materializes, it would virtually guarantee that ITER would be built, says Keilhacker. **D. B.**

► The report's call for more attention to be paid to ICF reflects a wider concern that Europe's fusion strategy may be off course. It asserts that "the weight and scope of the EU Fusion Programme" makes necessary a "thorough review of the scientific, technical and socio-economic prospects of its future R&D goals".

Indeed, parts of the report represent a thinly veiled attack on ITER. One member of the working group points out that, although the language of the report is polite "for the insider there are some very critical question marks about ITER".

This reflects growing concern among some fusion researchers that ITER may not achieve its stated goals. To meet these, ITER's performance would need to fulfil highly optimistic predictions, even though its operation would rely on several untested technologies. Any shortfall would result in a conspicuous and expensive failure that could set back support for fusion by decades.

Hans Karow, the secretary of the working party, is himself sceptical that ITER would achieve ignition. "It does not make sense to enter the pseudo-technical colossus of ITER before the physics case has been solved," he says. The report claims that inertial confinement "might still emerge as a superior way towards fusion energy".

Indeed, inertial confinement could in theory fuse deuterium with deuterium. This provides it with a big potential advantage over magnetic confinement, which can only fuse deuterium with tritium, as it would both eliminate the need to use radioactive tritium, and avoid the production of high-energy neutrons that make the first wall of the tokamak radioactive and weaken its structure. The first wall of a tokamak at 10^7 rads per hour is the hottest radioactive working environment on Earth.

Similarly, Sir William Mitchell, a former head of Britain's Science and Engineering Research Council, and a member of the working party, claims that although MCF may "look like big engineering", it is still in the research phase. "If both magnetic and inertial confinement are at the research stage, we should complete this stage before any decision [on how to proceed] is made."

The European Commission is already due to carry out a routine review of fusion research later this year. But the commission has now also agreed to a broader review that would include comparisons of fusion energy with other sources of energy and assess technical and environmental problems. Members of the panel will be nominated within the next few weeks.

JET director Martin Keilhacker, agrees that pressure is growing for a review of ITER as now envisaged. But he argues that some of this pressure is merely the result of budgetary politics, particularly in the United States, and maintains that "the ITER objectives are the right ones, and now is the right time to build it".

Declan Butler

Biotech industry woos MEPs to ease regulatory burden

Munich. Europe's biotechnology industry, still smarting from last year's rejection by the European Parliament of an attempt to harmonize patent legislation, is making an unprecedented effort to engage politicians in a dialogue aimed at reducing regulatory burdens on the industry.

Despite a report from the consultancy group Ernst and Young last week, indicating a 20 per cent increase in the number of biotechnology companies in Europe last year, the industry argues that the gap between Europe and the United States and Japan is increasing.

The first of a proposed series of information-exchange meetings took place in Strasbourg last week between representatives of the biotechnology industry and members of the European Parliament — who, following the Maastricht Treaty, can effectively veto any legislation regulating or promoting the industry's activities.

The initiative for the meeting came from Christof Tannert, a German socialist member of the European Parliament (MEP). Tannert is keen to see the industry adhere to strict safety standards, and is seeking a permanent structure for such meetings similar to the European Energy Foundation set up in 1981 to promote dialogue between MEPs and the energy industry.

Much of the industry's concern is focused on the revised draft directive on patenting of biotechnological products, which was drawn

many MEPs oppose on ethical grounds.

The forum wants this latter issue to be "explicitly resolved" during discussion of the directive, because of the uncertainty over the scope of patent legislation. Indeed, the European Patent Office (EPO), which is independent of the commission, has temporarily stopped granting patents on living organisms following a ruling by its Board of Appeals in February last year that a transgenic plant represents a collection of new varieties so — because the European Patent Convention disallows patents on plant and animal varieties — is unpatentable.

Confusion about the interpretation of the convention, which includes continuing uncertainty about the fate of the patent application on the Harvard 'oncomouse', has helped to focus attention on the European Parliament. The parliament will hold a public hearing on the new directive in Brussels on 10 and 11 June, and is expected to give the commission's proposals a first reading in November.

As the revised draft directive was drawn up after extensive discussions with the parliament, commission officials are optimistic that it will be approved. But parliament's mood can change rapidly in response to unexpected events. And one such event, according to Peter Stevenson of Compassion in World Farming, a British pressure group opposing the oncomouse patent, is current concern about bovine spongiform encephalopathy and its association with feeding cattle, which are naturally herbivores, with sheep brain.

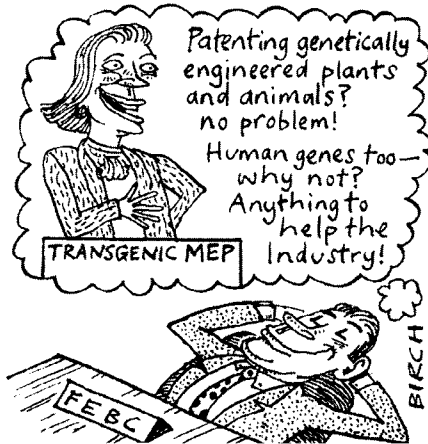
The unpredicted consequences of such "interference with nature", suggests Stevenson, "means that people are now much more ready to accept our arguments that genetic engineering of animals, and therefore their patenting, is wrong".

Industry is keenly aware of the parliament's sensitivity to public opinion. According to Peter Doyle, an executive director of the British life sciences company Zeneca, it is essential for industrial biotechnologists to remain in a "continuing dialogue" with Europe's elected representatives.

Doyle says that the European biotechnology industry needs a much more supportive regulatory environment. He agrees with some of the more positive signs described in the Ernst and Young report, but adds that "a small base growing at a rate of 20 per cent cannot catch up with a large base [as in the United States] growing at a slower rate".

He also points out that 26 of Europe's 28 publicly quoted companies are in the United Kingdom, where regulations are more flexible than other European countries.

Alison Abbott



up and approved by the European Commission last December after an earlier version had been rejected by the European Parliament (see *Nature* 374, 103; 1995).

The new directive, which has been approved by the Forum for European Bio-industry Coordination (FEBC), a Brussels-based umbrella group for industries with biotechnology interests, clarifies some of the issues that had concerned MEPs. But it still allows the patenting of human genes, as well as transgenic animals and plants, which

Drastic lay-offs loom at NASA headquarters

Washington. The US National Aeronautics and Space Administration (NASA) announced last week that it is to cut its headquarters staff by half over the next 18 months, a move that is expected to lead to the first forced lay-offs in more than 20 years.

The decision suggests that NASA is taking seriously new five-year budget projections handed down by the White House last month, despite recent public assurances by Daniel Goldin, the administrator of the agency, that the figures are not cast in stone.

In a memorandum to employees, Goldin cited "increasing budget pressures" as his reason for instructing the agency's Washington headquarters to reduce its staff from 1,400 to between 650 and 700 by 1 October 1997. NASA managers have identified 239 jobs that can be transferred to field centres; the rest of the cuts will have to come from voluntary resignations and lay-offs, with more recently hired employees facing the highest likelihood of losing their jobs. A final plan for the reductions, which are to be distributed evenly among the agency's programme offices, is expected early next month.

Goldin has long believed that NASA headquarters should be smaller and leaner, in line with practice in industry. The agency had already planned to cut its Washington office to a little more than 1,000 people by 2000. But the size and pace of the new cuts took NASA employees — including many senior managers — by surprise.

As recently as three weeks ago, Goldin told a congressional committee that he was planning "no precipitous action" on the basis of lower White House budget projections, which call for NASA funding to drop from \$13.8 billion next year to \$11.6 billion in 2000. Jack Gibbons, head of the Office of Science and Technology, has also been playing down the importance of any projections beyond 1997 (see box, right).

But others say that NASA — and indeed every federal agency — should take the new numbers seriously. "They undoubtedly are real," says Lori Garver, executive director of the National Space Society, and a member of NASA's advisory council. Garver says the council will be briefed this week on the long-term budget picture, expected to be grim.

Both the council and NASA's House of Representatives Appropriations Committee, which also meets this week, are expected to ask tough questions about the agency's ability to function with such a small headquarters staff. At a hearing last week, James Sensenbrenner (Republican, Wisconsin), who chairs NASA's authorization subcommittee in the House, continued to question whether the agency can still afford everything on its plate, given the reduced budget figures. This week, the House will release its own seven-year projections, expected to be

even harsher to federal agencies.

Senator Barbara Mikulski, whose Maryland constituency includes the Goddard Space Flight Center, issued an angry statement in response to NASA's action, calling



Head to head Goldin (left) and Mikulski disagree about the NASA proposals.

the proposed reductions "too much, too soon" and vowing to "stand sentry for the NASA budget in Congress" and to "take my fight all the way to the Oval Office if need be". But at the end of last week she was

alone among Washington-area representatives in speaking out against the cuts. Unlike the agency's field centres, headquarters employees have no vocal champions in Congress.

Last week's decision has heightened fears at the centres that they, too, face even greater layoffs in the near future than they had been led to believe. And for headquarters employees — who had been told repeatedly that such deep cuts were not in prospect — morale has not been so low since the Challenger accident, said one observer. While the headquarters library began setting out literature on how to find a job, an unofficial "RIF [Reduction in Force] Watch" home page popped up on the World Wide Web, with a picture of a rocket spinning out of control and an acerbic quote: "Gee, we waited 'til the last minute, removed one out of every two components, and left the oldest ones in. I can't understand why it failed."

Tony Reichhardt

Budget projections 'nearly meaningless'

Washington. Jack Gibbons, President Bill Clinton's science adviser, has told US science lobbyists not to be too concerned with medium-term budget projections, and to concentrate instead on Congress's plans for cuts in the 1997 financial year, which starts on 1 October.

"The bottom line is that we are in the middle of a crisis this year and this month," Gibbons told the annual science policy symposium of the American Association for the Advancement of Science (AAAS) in Washington last week. "I don't know what 2002 will bring — neither does the Congress, neither does AAAS."

Gibbons was responding to an assessment by the AAAS of the Clinton administration's own budget proposals which had pointed out that the proposals would result in cuts in the science budget by 12 per cent by the year 2002, with very sharp cuts during 1998–2000 (see *Nature* **380**, 572; 1996).

Administration officials have repeatedly sought to play down the significance of their figures for the so-called 'out-years', and Gibbons told the AAAS that "the numbers and assumptions" behind the projections "border on the nearly meaningless".

"I'll be happy to have a debate over the 'out-years' when we know more about them," Gibbons said. Focusing on the situation for the fiscal year 1997, he added: "I want to point out the differences between President Clinton's commitment to science, and the seeming disdain some in Congress display for the

increasing knowledge base that research makes possible — differences all too easily obscured by our obsession with numbers on a ledger sheet."

But the power of the ledger sheet was all too apparent on the day he spoke, when NASA announced hundreds of compulsory redundancies at its headquarters (see above), after struggling for years to avoid such redundancies.

Furthermore, physics lobbyists at last week's AAAS meeting said that the Department of Energy is already drawing up its 1998 budget proposals on the basis of the medium-term projections in the Clinton budget.

These projections include an 8 per cent cut in the \$1-billion general sciences budget at the energy department, which funds most particle and nuclear physics in the United States. A senior department official confirmed that it was planning around these projections, but said this did not necessarily mean that the projections would be implemented.

A statement issued by Gibbons in direct response to the AAAS assessment pointed out that Clinton has called for increases in science and technology funding for four years in a row. "The best way to predict the future is to look at what we have done in the past," said the statement, adding that the AAAS appeared to have underestimated future research and development budgets by assuming that cuts in federal agencies will hit all programmes equally hard.

Colin Macilwain

Block grant opposed for NIH clinical centre

Washington. A leading congressional figure responsible for handling the budget of the US National Institutes of Health (NIH) said last week that he will not support President Bill Clinton's request that all the funding for the new \$310-million Clinical Research Center (CRC) should be taken from the 1997 budget.

John Porter (Republican, Illinois), chairman of the House of Representatives appropriations subcommittee that oversees NIH spending, said that, in the absence of an unexpectedly large — and unlikely — budget allocation to his committee, he plans to propose that the money for the centre be distributed over three or four years.

Porter said that he would prefer the budget for the fiscal year 1997, which begins on 1 October, to fund merely "one of the four years" for the centre. This would then allow his subcommittee to fund extramural research "at an increased level of, say, 6 or 6.5 per cent [above the 1996 level]" in 1997. The current budget proposal allows for an increase of only 2.6 per cent in extramural research grant funding.

Porter, speaking after a hearing of the

subcommittee on the NIH's budget request, argued that, because extramural research needs "momentum", it would suffer without a larger increase than the president proposed. "If you squeeze it down in the way that the [Clinton] administration suggests, I think you're going to lose a great deal more than just what the numbers might suggest. You are going to lose people. You are going to lose commitment."

The Office of Management and Budget (OMB) maintains that allocating all of the money in a single year will be more efficient. Lawrence Haas, a spokesman for OMB, says that it also avoids the risk that Congress will cut off funds in later years, "which would leave you with a half-built building".

But Porter argues that there is virtually

no risk of such an outcome. "The potential for finishing [the construction work] is very good. It's very unlikely we would back away from that commitment in future years".

At the hearing last week, Porter quizzed Harold Varmus, the director of NIH, on the impact of compensatory cuts in other NIH programmes in the Clinton administration's 1997 budget proposal, released in March. About two-thirds of the \$467 million in new money requested for the NIH — a 3.9 per cent increase over 1996 — would be used to finance the new clinical facility. Most of the remainder would go to extramural research grants. But funding for intramural research would fall by 0.2 per cent, and multi-year extramural grant recipients would fall from four to two per cent in their routine increases to cover the costs of inflation. ('Biomedical inflation' is now 3.7 per cent).

The CRC is a planned 250-bed addition to the current NIH Clinical Center, a 1953 building that would be partially converted to other uses. Completion of the extension is expected early next century. Varmus argued that money being requested for the new facility is an "add-on", not a substitute for support for external scientists.

Varmus also said that the reduction in inflationary increases for multi-year grant recipients would not impose a significant burden. "This is a very tight budgetary environment. And the money [for the CRC] is being requested in response to a clear need," he said.

But Francine Little, director of the NIH's Office of Financial Management, told scientists at a separate meeting last week that the A111 had initially proposed that the CRC be funded in \$90million installments over four years. The Clinton administration modified the request to a single allotment.

This proposal has caused murmuring among extramural researchers and their allies in Congress. It leaves "only enough money for about a 1.6 per cent increase for everything else, particularly research grants," says Jerold Roschwalb, a lobbyist for the National Association of State Universities and Land-Grant Colleges, which represents major public research universities in every state.

Porter, an ardent advocate for biomedical research, appears to have lost none of his enthusiasm for the NIH since last year, when he was primarily responsible for securing a 5.8 per cent budget increase for the NIH in 1996 at a time when other federal agencies were limping along on temporary spending measures or being forced to shut down due to a budget impasse. "I just can't think of any part of government that is doing a better job," he told Varmus. "We just want to do everything we can to provide to you the kind of tools that you need including the clinical centre."

Meredith Wadman



Porter: prefers phased funding for CRC.

Chernobyl damage 'underestimated'

Munich & Tokyo. The medical consequences of the Chernobyl disaster have been seriously underestimated, according to Alexei Yablokov, head of the Centre for Russian Environment Policy, who chaired a meeting in Moscow last week attended by 45 non-governmental organizations.

Yablokov criticized Western scientists for ignoring data gathered by scientists from the new independent states (NIS) of the former Soviet Union. So far there is only a consensus on an increase in thyroid cancer in children, but NIS researchers presented extensive data on alterations in biological parameters at the cellular, biochemical and immunological levels in populations exposed to low levels of radiation for prolonged periods. They also presented epidemiological studies reporting increased incidences of a range of ailments.

Such results are regarded with caution by Western scientists because of the lack of controls and uncertain diagnoses. John Harrison, for example, deputy director of the UK National Radiological Protection Board, who has worked with the World Health Organization (WHO) programme to improve methods of collecting data in the affected areas, says one problem with the NIS data is the lack of a reliable registry of mortality and morbidity in the affected states (Belarus, Russia and Ukraine).

But according to Yablokov, who was science adviser to Boris Yeltsin, the health effects of Chernobyl will plague its victims

"for generations, practically forever". Low levels of radiation have been shown to have a much greater effect than predicted from the accepted view that dose-effect relationships are linear, he claims.

Nevertheless, there is broad agreement on the need for continued monitoring of the health of those affected by radiation. Because of the long latency of most solid tumours, says Harrison, no firm conclusions about the health consequences of the accident can be made for at least ten years.

But it is not clear how long international funding is likely to continue. In addition to bilateral health programmes, in particular the Japanese Sasakawa Memorial Health Foundation, which provided more than ECU40 million (US\$50 million) to set up five medical diagnostic centres within the affected areas, the European Commission (EC) and the World Health Organization (WHO) have been the major sponsors of international health programmes, which are now coming to an end.

Nearly all of the WHO money was donated by Japan, and the organization has not yet succeeded in persuading any country to finance a continuation of the programme. The EC will continue supporting projects within the fourth Framework programme, which runs until 1999, but these will now have to compete for the general pool of money set aside for research involving NIS partners, the so-called INCO programme.

Alison Abbott & Stephen Barker

US broadens scientific base of controls on carcinogens

Washington. The US Environmental Protection Agency (EPA) this week unveiled the first revisions to its guidelines for determining human cancer risk in ten years. The long-awaited guidelines will allow more flexibility in regulating carcinogens, and will ensure that "cancer risk assessments reflect the very best scientific knowledge available", according to Carol Browner, the EPA administrator.

In doing so, the new guidelines in effect shift decision-making away from scientists and toward 'risk managers'. But some environmentalists fear that they will also lead to more ambiguous risk assessments, and make it more difficult to regulate suspected carcinogens. "From a public policy standpoint, we are extremely disappointed," says Al Meyerhoff of the Natural Resources Defense Council (NRDC). He calls the new guidelines "an invitation to torpor, delay and foot-dragging".

Traditionally, EPA has relied heavily on rodent bioassays to establish whether a particular agent causes cancer. Rats are given a chemical, and then watched for two years to see if tumours develop. In contrast, the new guidelines call for using a wider range of information, including genetic data, information on the agent's physical and chemical structure, and testing for biological effects leading up to tumour development.

EPA has been making increasing use of these other techniques in recent years to assess cancer risk. But they often have been a "secondary thought" says Jeanette Wiltse of the agency's National Center for Environmental Assessment in Washington.

Under the revised guidelines, EPA will drop its long-standing scheme for classifying cancer hazards by assigning each substance to one of six categories, starting with Category A for known human carcinogens. Instead, the agent will be classified according to one of three 'descriptors' of its human carcinogenic potential: 'known/likely', 'cannot be determined', and 'not likely'.

Each substance also will receive a narrative description of its potential to cause cancer under different conditions, as well as a short summary of the animal and human evidence supporting these conclusions. These 'weight of evidence' narratives are meant to give risk managers more information than just a simple category designation, says Wiltse. They will also allow risk assessors "to express things they hadn't been expressing when they had to boil [the classification] down to a letter".

Gone, too, is EPA's previous assumption that all dose-response relationships are strictly linear. Risk assessors may now use non-linear approaches to estimating toxic

effects at low doses, which could lead to known carcinogens being declared safe below a certain threshold. The new guidelines also allow risk assessors to consider different exposure routes — for example, inhalation versus ingestion — as well as effects on different populations, such as children and pregnant women.

The proposed new guidelines closely follow recommendations from a 1994 National Research Council (NRC) study *Science and Judgment in Risk Assessment*, and the initial reaction to EPA's announcement among scientists has generally been favourable. Ronald Estabrook of the University of Texas Southwestern Medical Center in Dallas, for example, who chaired a recent NRC study of carcinogens in food, calls the added flexibility "a very positive move".

Roger McLellan of the Chemical Industry Institute of Toxicology in North Carolina says that he particularly supports EPA's shift from a single category designation to a narrative description of cancer hazards. This, he says, "recognizes that chemicals can cause cancer in different ways" and gets away from the "tyranny of lists".

But Meyerhoff says his organization opposes the shift away from "the bright red line we've had in the past" to differentiate between "known" and "probable" carcinogens, and adds that NRDC will seek to retain the old category system. He also says that the guidelines, in their increased reliance on new types of information, overstate the ability of scientists to understand the causes of cancer. "I fear we're seeing scientific hubris here," he says. "These guidelines assume far more certainty about carcinogenesis than actually exists."

EPA says it plans to continue using existing cancer assessments until revised assessments based on new data are completed and have been peer-reviewed. That raises another fear among environmentalists — that chemical manufacturers will flood the agency with requests to reassess any substances now labelled carcinogenic, and paralyze the process as a result.

But Wiltse says that in some cases, new techniques can speed up the process, particularly when risk assessors do not have to wait for two-year rat tumour studies to be completed. Substances that already have a large database and an advanced risk assessment will move more quickly through the reassessment process, she says.

The proposed guidelines are being published in the *Federal Register* this week, and have been posted on the World Wide Web at <http://www.epa.gov/ORD/WebPubs/carcinogen>. The public will have four months to comment.

Tony Reichhardt

IMAGE
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REASONS

Yeltsin: reaching out for scientists' votes.

Yeltsin promises more money for science

Moscow. President Boris Yeltsin, currently campaigning for re-election, last week signed a decree promising the Russian Academy of Science a budget next year that is 50 per cent higher than this year's, granting additional tax privileges to the academy, and allocating 50 billion rubles (US\$10 million) a year over the next five years for the construction of apartments for young scientists.

The barely concealed political motivation behind the move became even more obvious the following day, when Yeltsin made a speech in which he said he relied strongly on scientists and was eager to support them. "Only science and education can prevent a political and social Chernobyl, since the chain reaction of the former regime's disintegration has not yet been stopped," he said.

Yeltsin was taking part in centenary anniversary celebrations for Nikolai Semenov, the first Soviet winner of the Nobel prize in 1956 for his study of chemical reactions, held in the Grand Hall of Columns in Moscow.

The prime minister, Viktor Chernomyrdin, presided over the meeting, which was also attended by Federico Mayor, the director general of the United Nations Educational, Scientific and Cultural Organization (Unesco).

Also present were deputy prime minister Oleg Soskovets, Evgeny Primakov, the foreign affairs minister, Yuri Luzhkov, the mayor of Moscow, and Yuri Osipov, president of the academy. Their combined presence at a scientific gathering, virtually without precedence in the past few decades, was seen as a clear signal to the scientific community that Yeltsin and his supporters are seeking their support in the coming presidential elections.

Mayor told the Russian officials present that he was certain that the contribution of Russian scientists to world science "will be proportional to Russia's contribution to international cooperation" — a comment which was seen by some as an indirect reference to the fact that Russia still owes money to Unesco, although others claimed that it was merely a standard piece of public rhetoric.

Carl Levitin

Interest ferments in yeast genome sequence

Paris. The first complete sequence of the genome of a eukaryote, the yeast *Saccharomyces cerevisiae*, will be achieved this week with the release of the final portion of the sequence by the European-led consortium of 96 laboratories — including some in the United States, Japan and Canada — involved in the Yeast Genome Project.

The yeast genome is made up of 12.06 million bases distributed among 16 chromosomes, and contains around 6,000 genes. It is about 250 times smaller than the human genome. The availability of the entire yeast genome will provide unprecedented information about the architecture of a eukaryote genome, according to André Goffeau of the Université Catholique de Louvain in Belgium, who is the coordinator of the Yeast Genome Project.

This has already shown that the yeast genome is much more densely covered with genes than expected, says Goffeau, while much of it is also redundant, with repeated sequences on various chromosomes. Waves and troughs of G-C rich and poor areas also occur on all the chromosomes. Both features must have evolutionary significance, he adds, while the G-C waves may also be

IMAGE
UNAVAILABLE
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REASONS

Brewing up: sequencing of the yeast genome follows the first genetically engineered beer recently launched in Britain.

important for replication. The sequence of the telomeres of all the yeast chromosomes is also unexpectedly similar.

The complete inventory of the yeast genome has also revealed a surprising number of both unknown genes — amounting to around one-third of the 6,000 genes identified in its genome — and new members of known protein families. For example, more than 20 putative sugar transporters, identified on the basis of their

James King-Holmes/SPL

transmembrane spans, have been found.

Clusters of proteins with similar structures, but no homology to known proteins, also occur. "We are discovering that there are many more protein families than we thought," says Goffeau. Discovering the function of these unknown proteins will be the aim of a new European Union programme, EUOFAN, which will receive ECU7.7 million (US\$9.6 million) over the next two years. The programme will involve 144 laboratories.

The level of homology of yeast genes with human genes is also "striking", says Goffeau, who predicts that as many as half the yeast genes will be "significantly similar" to human genes. Many code for proteins involved in human diseases, and may open the way for the mechanisms of such pathologies to be studied within yeast, a much simpler alternative to doing so in human cells.

Bruno Hansen, head of life sciences at the European Commission, and former vice-president of research at Novo Nordisk, predicts that this high level of homology also means that the results of the yeast genome project will play a major role in underpinning progress in the understanding of the ►

AIDS advisers disagree over events in HIV blood scandal

Tokyo. After the Japanese government's recent admission of its failure to prevent the infection of thousands of haemophiliacs with HIV in the early to mid-1980s, two parliamentary committees last week heard testimony from three scientists involved in deciding Japan's policy on blood products.

Their contradictory testimony, presented to the health and welfare committees of the upper and lower houses of Japan's Diet, raised more questions than answers. But it did provide some insight into the decisions and actions of these policy-makers.

The three were Takeshi Abe, former vice president of Teikyo University, who headed an AIDS study group established by the Ministry of Health and Welfare in 1983; Atsuki Gunji, a professor at Tokyo University medical school, who headed the ministry's biologics and antibiotics division from 1982 to 1984 and setup the study group; and Juzo Matsuda, an assistant professor of Teikyo University, who was a member of the study group.

It was this study group and its subcommittee that recommended in 1983 that haemophiliacs should continue to use blood coagulants that had not been heat-treated to kill viruses, and which is said to have rejected a proposal for emergency imports of heat-treated products. Instead, clinical trials of heat-treated products were recommended and were carried out between 1984 and 1985, coordinated by Abe.

Abe and Gunji, who testified separately and have previously given conflicting accounts of the events in question, failed to agree on the purpose or role of the AIDS study group. Abe claimed that its role was chiefly to determine whether there were any AIDS patients in Japan, and insisted that the group had only an advisory academic and scientific purpose and played no part in setting administrative policy.

But Gunji argued that the group's purpose was to examine how haemophiliacs were being treated and to decide if any changes were necessary because of the AIDS epidemic. He added that, while the group was set up only with research funds and was not intended to have an administrative role, it ended up becoming a "very important decision-making" body.

Matsuda testified that the study group's decisions resulted in the spread of HIV being much wider and he apologised to haemophiliacs. But Abe refused to take any responsibility, saying that "there is no point in feeling responsible".

Abe was repeatedly asked at both hearings about allegations that he deliberately delayed clinical trials of heat-treated blood coagulants so that the Japanese blood product manufacturer Green Cross Corporation (Midori Juji) could catch up with US and European manufacturers in the development of such products. He was also quizzed about large donations that he received from

Midori Juji and other companies in the clinical trials for a foundation that he set up.

Abe said it was "ridiculous" to suggest that he delayed the clinical trials. He claimed that he received money from the companies, ¥10 million (about US\$100,000) each, only between May and July 1983, before the clinical trials started. He said it was "insulting" to suggest any link between the donations and the trials.

But Gunji testified that in "late 1983" he received several complaints from foreign blood product manufacturers that Abe was requesting money, and that these had asked if such behaviour was acceptable. Gunji said he therefore decided to "warn" Abe about his fund-raising.

Although it was not discussed at the hearings, it has been widely reported that Abe, angered by Gunji's warning, suspended organization of the clinical trials and their start was delayed for a month until February 1984 because Abe, at the time Japan's leading authority on haemophilia, was seen as the only person with the power and influence to assemble the necessary groups of patients for the trials.

Abe did, however, admit in his testimony that he urged Midori Juji, Japan's largest blood manufacturer, to make heat-treated products "as quickly as possible". He said that this was because the clinical trials could not be carried out without Midori Juji's products.

David Swinbanks

►human genome. Indeed, Hansen says that the completion of the yeast genome will have a broad impact on biological research and development. "It has been money well spent for Europe's taxpayers," he says.

The European Union is also supporting the sequencing of another yeast, *Schizosaccharomyces pombe*, which has just three chromosomes. Scientists are looking forward eagerly to comparing the genomes of the two yeasts, as *S. pombe* is in some ways closer to humans than *S. cerevisiae*.

The completed sequencing of the yeast genome follows that which has already been accomplished of two prokaryotes, the 1.8 MB genome of *Haemophilus influenzae* and the 0.58 MB of *Mycoplasma genitalium*.

Few deny that the completion of the yeast genome represents a major success for Europe, and vindicates its strategy of distributing the work among many laboratories in a tightly coordinated way. The sequencing of the first chromosome of yeast — chromosome III — in 1992 was accomplished by each European laboratory sequencing a predetermined portion of the chromosome. At the time, it was the first chromosome to be sequenced and the largest continuous sequence of DNA known (see *Nature* 357, 38; 1992).

This approach was subsequently extended to the whole genome, with individual chromosomes being attributed to groups of 15–20 laboratories, which in turn divided the work among themselves. All data was centralized at the Martinsried Institute for Protein Sequence in Germany.

But the efficiency of this network model cannot be measured simply by the rate of sequencing, says Hansen. He points out that the large number of scientists involved are not merely engaged in sequencing but are also carrying out their own research, thus multiplying the benefits. "Scientists at centralized sequencing facilities can be too removed from the real world of research", he claims.

Declan Butler

MRC faces negligence claims over growth hormone victims

London. Britain's Medical Research Council (MRC) was in court last week facing allegations of negligence in the experimental treatment in the 1960s and 1970s of children using human growth hormone (hGH) from the pituitary glands of people who had died.

The programme was discontinued in 1985, after three patients who had undergone the treatment in the United States died from Creutzfeldt-Jakob disease (CJD). Later that year a new technique for manufacturing hGH by genetic engineering became available.

The High Court in London has now opened a hearing on eight of 17 cases of CJD contracted by individuals in the United Kingdom treated with hGH before 1985. All but one of the 17 have since died.

Families of the eight victims started legal action against both the MRC and the Department of Health after the latter turned down calls for a public inquiry. At issue is whether the MRC and the department owed a duty of care to the children undergoing hGH treatment and, if so, whether they were in breach of that duty.

The families allege that the two bodies were negligent in not taking into account evidence that CJD was transmissible, not ensuring that no pituitaries were taken from people suffering from neurological disease, and not using the safest method of extraction of the hormone.

The MRC began the hGH programme in 1957 and ran it until the Department of Health took over control in 1976. But the MRC continued to manufacture the hormone until 1980, while the Department of Health was setting up production facilities at the Centre for Applied Microbiology and Research at Porton Down.

Between 1957 and 1980 the only source of the hormone was the pituitary glands of people who had recently died. "Demand for the human growth hormone increased because it was a successful treatment," says a spokesman for the MRC. Nearly 2,000 people received the hormone treatment.

Over the period when hGH was extracted from cadavers, nearly one million pituitary glands were used in the United Kingdom. Mortuary attendants removed the glands and at one stage "received a nominal fee of twenty pence per gland". This system was later changed and mortuary attendants were paid a flat rate for the extra work involved in removing the glands.

According to Hugh Fraser, former head of the Neuropathogenesis Unit at the Institute for Animal Health in Edinburgh — and an expert witness in the court case — a critical question the judge will have to decide is "what knowledge was in place and when".

As early as the late 1950s, it was recognized that scrapie-like diseases (such as CJD) might be transmissible. Later it was shown that biopsy material taken from the brain of a patient with CJD induced a similar brain disease when injected into a chimpanzee.

Such information raised warning signals among researchers. In October 1976, for example, Alan Dickinson, then head of the Disease Studies Unit at the Agricultural Research Council's laboratory in Edinburgh, says he telephoned the MRC to inform them of potential risks in the procedure.

A spokesman for the MRC said last week that when the first evidence suggested the possibility of potential risks, it was investigated. "When the evidence became substantive, they stopped using it," he added.

Richard Nicholson, editor of *The Bulletin of Medical Ethics*, points out that doctors providing a drug or a hormone as a treatment have a duty to inform their patients of any known side-effects. "The question is, at what stage did the MRC know about these risks?", he says.

While acknowledging that contaminated hGH was the source of the CJD infection, the Department of Health and the MRC deny negligence. In a statement issued last week, the MRC says that many people recognize that no form of medical treatment is without risk, and denies that it was negligent in supporting and monitoring the trial.

Irwin Mitchell, the solicitors for the plaintiffs, say that the court's judgement will have a direct effect on a further 200 cases now pending that have been filed on behalf of those who have not developed CJD as a result of hGH treatment, but fear that they may do so.

Ruth Bell

Italian researchers look for return of Ruberti

Rome. Sunday's general election in Italy seems to have brought good news for Italian researchers, who are optimistic that they will find the new centre-left government more sympathetic to the needs of science than its recent predecessors — and the familiar figure of Antonio Ruberti once again as research minister.

Romano Prodi, the new prime minister, who is professor of economics at the University of Bologna, has previously said that he ranks science and higher education among his priorities (see *Nature* 375, 620; 1995). The quality of science in Italy is patchy, he said last year, and this must change. During the election campaign he named Ruberti as official candidate for the research minister post.

Ruberti, who is 69, will bring much-

needed experience to the job. He was formerly Italy's research minister for the five years up to 1992, during which time he introduced some important legislation, including laws on the autonomy of universities. He then served for two years as European Union research commissioner.

Ruberti is well known as a supporter of basic research, as well as a believer in the importance of European collaboration. He says that, if appointed, he is keen to install appropriate structures for quality control of Italian research, and to increase the level of university autonomy

A. A.



Ruberti: heading for government?

Controversial AIDS vaccine fails to delay disease

Washington. The results of a five-year US study announced last week show that an experimental AIDS vaccine based on the genetically engineered HIV envelope protein gp160 does not affect the course of disease in HIV-infected volunteers.

The study, conducted under the auspices of the Walter Reed Army Institute of Research in Washington DC and the National Institute of Allergy and Infectious Diseases, followed 608 volunteers for five years, until December 1995. Half were given the gp160 vaccine and half a placebo.

The study found that the vaccine, produced by Microgenesys Inc. of Meriden, Connecticut, led to an elevated immune response in those injected, but did not slow progression of the disease. "No clinical improvement" could be attributed to the vaccine, used as adjunct therapy for HIV infection, said a statement released by the Department of Defense.

The gp160 vaccine was at the heart of a controversy in 1992, when former Senator Russell Long (Democrat, Louisiana), a lobbyist for the company, helped it to land \$20 million in Defense Department funding for a large trial of the vaccine. □

Ebola virus prompts monkey ban

Washington. The Philippines has banned the export of live monkeys after the death of two monkeys from an Ebola-like virus in a breeding facility in Texas. The monkeys had been imported from the Philippines last month. The ban will remain while a task force carries out tests on monkeys in Philippine breeding farms.

According to US and Philippine officials, the strain of the virus, known as Ebola Reston, poses no threat to public health. A third

monkey at the Texas centre has been infected with the virus, and, along with 47 others, will have to be destroyed, Texan health officials confirmed. The Philippines exports about 2,500 monkeys a year for medical research to the United States, Japan and Europe. □

Germany shifts polar research ...

Munich. The German government last week announced a change in orientation of its polar research programme, which receives DM82 million (US\$59.2 million) a year. In line with decisions at the 1992 climate conference in Rio de Janeiro, Germany will concentrate its research on climate issues, including study of the ozone layer above the South and North Poles. For the first time, German scientists will work in Russian research stations in Arctic regions. The programme will also support studies on polar ecological systems. □

...and clears biotech programme

Munich. Bavaria last week approved a DM7-million (US\$4.8-million) biotechnology research programme to succeed its four-year DM5.3-million *Forbiosich* (Research into Biotechnology Safety) programme, which ends in December. The new programme will run for three years and will support a broader research base than its predecessor, which was restricted to retroviruses. Both programmes are supported by the Bavarian Research Foundation, established in 1993 with money from the state's extensive privatization programme. □

Spending cuts 'undermine revival'

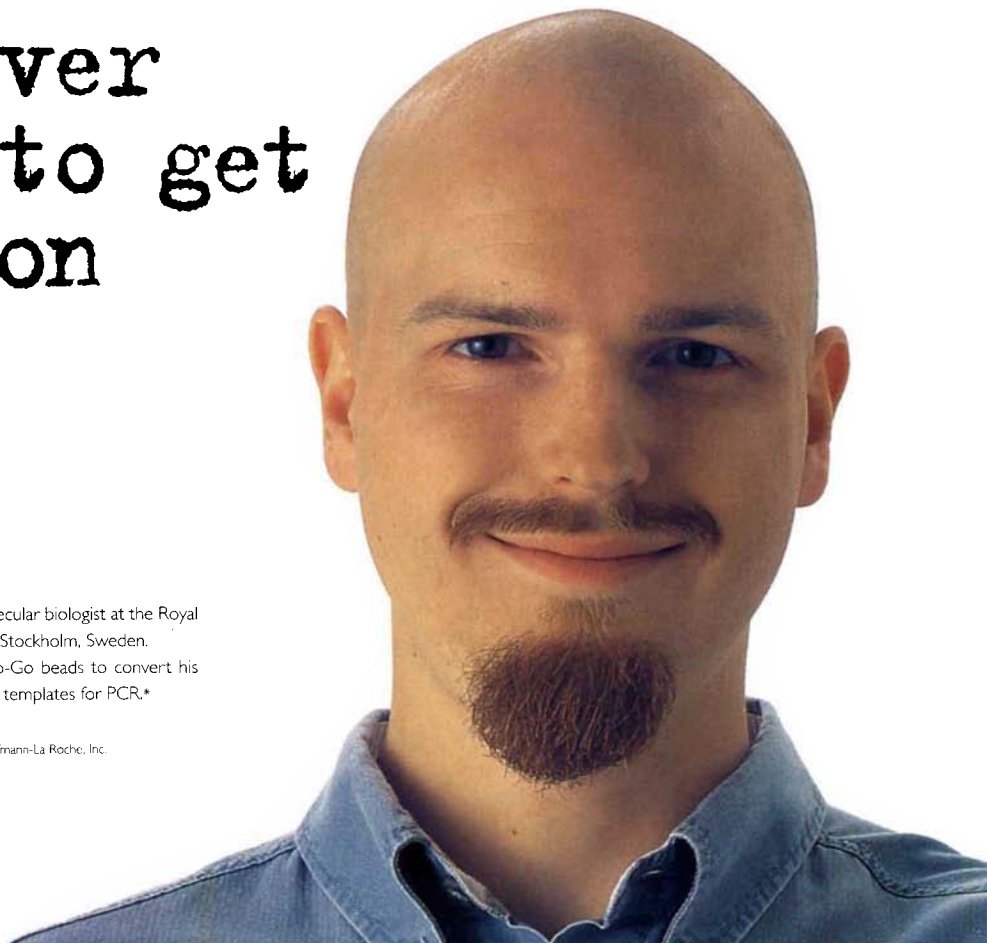
Paris. Massive cuts in spending on science and technology in the countries of the former Soviet Union risk undermining the market-oriented research and development base needed for economic and social revival, according to a paper published this week by the United Nations Educational Scientific and

Patrik never fails to get a reaction

Patrik Samuelson is a molecular biologist at the Royal Institute of Technology in Stockholm, Sweden.

Patrik uses Ready-To-Go beads to convert his RNA samples into cDNA templates for PCR.*

* PCR is a patented process of Hoffmann-La Roche, Inc.



Cultural Organization as part of its *World Science Report 1996*.*

The paper, which was written by Leonid Gokhberg — deputy director of the Moscow-based Centre for Science Research — points out that most industrial research and development was military oriented, but that the huge cuts in defence spending are being made too quickly to allow its conversion to civilian ends. As a result, many large research institutes now face closure, he claims, while the links between research and industry that are needed for economic revival risk being irreversibly broken.

**World Science Report 1996*, Unesco Publishing, Paris (h.moore@unesco.org), ISBN 92-3-103220-8. □

Sandoz to buy organ company

London. The pharmaceutical company Sandoz Pharma Ltd announced last week that it is to purchase Imutran, a privately held biotechnology company that specializes in the development of animal organs for transplantation. Imutran last year announced that it had developed a technique for overcoming hyperacute rejection, the main barrier to xenotransplantation, which can occur within minutes or hours of transplantation (see *Nature* 377, 185; 1995). □

Light sentences for DNA objectors

Washington. Two US marines court-martialled for refusing an order to provide blood and tissue samples for a Department of Defense gene bank have received nominal punishments, being confined to base for seven days and given a written reprimand.

The two — Lance Corporal John Mayfield and Corporal Joseph Vlacovsky — could have faced a dishonourable discharge and up to six months imprisonment (see *Nature* 380, 570; 1996). The Council for Responsible Genetics, a lobby group supporting the pair, said that in choosing the light sentences, “the military judge recognised [the] substantial and legitimate concerns” motivating the marines. □

White House nominates officials

Washington. The White House has selected nominees to fill two key posts at its Office of Science and Technology Policy (OSTP), according to administration officials. But it is unclear whether the Senate will ratify the choices before November's presidential election.

Jerry McIlillo, a prominent biogeochemist from the Marine Biological Laboratory at Woods Hole, Massachusetts, is the White House's choice as associate director, environment, subject to security clearance. He would replace Bob Watson, who is moving to the World Bank next week.

Kerri-Ann Jones, an OSTP official who previously worked with the Agency for International Development, will be nominated as associate director, national security and international affairs. □

Science camp exclusion claim

Washington. The US National Science Foundation (NSF) and Texas A&M University have settled a legal case brought last year by a white, 12-year-old girl excluded from an NSF-sponsored summer camp designed to foster interest in science among disadvantaged students (see *Nature* 374, 394; 1995). Under the settlement, the NSF has promised that its summer science camps will be open to students of all races, and has paid \$5,000 of the unnamed plaintiff's costs. □

How much vitamin C is best?

Washington. The optimal daily intake of vitamin C is about 200 mg, not the thousands of milligrams that the Nobel prizewinning chemist Linus Pauling believed would provide protection against serious illnesses, according to a study by the National Institutes of Health. The study, published in the *Proceedings of the National Academy of Sciences*, concludes that daily doses above 400 mg have no evident value, and that 1,000 mg or more could be hazardous. □

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'Gene hunting' in India

SIR — Your recent News stories about 'gene-hunting' in India (see *Nature* 379, 381–382; 1996) refer to a memorandum published by the Indian government's Ministry of Health and Family Welfare in 1992. This points out that the government does not object to biological samples being sent abroad for diagnostic purposes for studies that either cannot be done, or require research techniques that are not available, in India. Where the studies can be carried out in India, any proposal to send biological products abroad for such studies "should be sent to the Director-General, Indian Council of Medical Research, New Delhi, who will be the nodal point to clear all such proposals". The material sent abroad from the eye hospitals cited in the report was for collaborative research work, and therefore, despite the suggestion in your article, meets the requirements of regulations.

Furthermore, both the L. V. Prasad Eye Institute (LVPEI) in Hyderabad and the Sankar Nethralaya (SN) in Madras have tried unsuccessfully to set up joint research projects with Indian geneticists and basic researchers. Given the urgency and the magnitude of the problem of retinosa pigmentosa in India, the worldwide prevalence of the disease, and the necessity for comparative studies, they sought out foreign collaborators, and the same is probably true of the other eye hospitals mentioned in the report.

The eye institute says that the funds it has received for the blood samples merely cover the reimbursement of the costs of clinical analysis, patient care and food and transportation, amounting to less than US\$20,000, and denies that it is to receive US\$180,000 for the future supply of human blood.

Finally, I should like to add that international collaboration between medical researchers, including collaboration that requires the exchange of genetic material, is a noble, uncommercial activity. It needs to be emphasized that the issues raised in your articles are qualitatively different from concern about patenting, licensing and intellectual property rights concerning medicinal plants, seed and other agricultural materials.

D. Balasubramanian

(Director)
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and Molecular Biology,
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SIR — It is sad to read the criticism being levelled against the 'illegal' export of human DNA and blood samples from India for medical research on the grounds that it represents a plunder of India's

genetic material.

Many important human genes for inherited diseases have been discovered in recent years, each involving several hundred person-years of research work with patients' samples collected from dozens of hospitals across the globe. For India to participate in such collaborative ventures does not represent pillage, but a creditable achievement.

Furthermore, research laboratories in the developed world are often criticized for neglecting problems relevant to developing countries. To complain at the same time that human biological samples are being plundered from the latter is therefore self-contradictory, as it is obviously impossible to work on diseases such as giardiasis or leprosy without appropriate material from patients. 'Gene-hunting' research is no different, either conceptually or operationally, from that on infectious diseases.

There is a question of human rights. The genetic material referred to is associated with the right of individuals to decide whether to provide their informed consent to particular forms of treatment, including research into the disease from which they suffer. Any government regulations that prevent such individuals from making a free choice in such circumstances would appear to violate a fundamental right, and are therefore unconstitutional. The individuals in question approached the clinics voluntarily, seeking relief from their ailments; experience worldwide has shown that such individuals often express keen interest in cooperating with researchers attempting to trace the cause of their ailment. An incurable disease such as retinitis pigmentosa is devastating for its victims. Any research into its causes, even if it transcends national boundaries, should be praised rather than criticized.

If the current debate eventually codifies the way in which appropriate credit, both intellectual and financial, is allocated to Indian participants involved, through the identification of affected families, in any new discovery that emerges, it will have served its purpose. But if the debate merely results in the cause of diseases remaining undiscovered for decades to come, it will be a distressing outcome, not least for the patients concerned.

J. Gowrishankar

Centre for Cellular
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SIR — One of the current aims of the United Nations Educational, Scientific and Cultural Organisation (UNESCO) is to give developing countries an opportunity to participate in the human genome project. At the request of Indian colleagues at the

third Unesco South-North Human Genome Conference, cosponsored by the International Centre for Genetic Engineering and Biotechnology, in New Delhi last December, an *ad hoc* committee drafted a statement.

This pointed out that scientists attending the meeting had acknowledged the need for the dissemination to the public of accurate and understandable information about the medical and scientific benefits that will emerge from increasing knowledge of the human and other genomes. The application of such knowledge has already made significant contributions to the fields of medical diagnosis, therapy and agriculture. At the same time, the appropriate use of the potential economic return from such biotechnological applications is of concern to both developed and developing countries.

The statement added that India's ethnic diversity and its large population is already contributing to knowledge about human genomic diversity. Indian scientists and their international collaborators support the goal that developing nations and individual ethnic groups should receive their appropriate share of the economic and commercial returns derived from medical investigations, including both clinical trials and genetic epidemiological studies, using biological material derived from their population groups.

Santiago Grisolia

(Chairman, UNESCO Scientific
Coordinating Committee for
the Human Genome Program)
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Public scepticism

SIR — Christopher B. Johnson's statement (*Nature* 380, 18; 1996) that "it is not immediately obvious why the principle of healthy scepticism of scientific truths should be perceived as laudable within the scientific community but as unfortunate within the population at large" sounds somewhat surprising. The explanation is simple: nowhere on Earth at any time in human history has "the population at large" been educated to appreciate and practise scepticism about alleged "truths", those of a religious and ideological nature in particular. The fate (even now) of bravely "misbehaving" individuals is well known. So how can one possibly expect people "at large" to be enlightened enough to applaud "scepticism of scientific truths"?

Helmut Grunewald

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Germany

Life in the hot zone around Chernobyl

David M. Hillis

A consequence of the Chernobyl nuclear accident is an increased rate of inherited mutation in mammals (including humans) living in the region. But the causes and the long-term effects on health remain unclear.

TOMORROW, the 26th of April, is the tenth anniversary of the world's worst reported nuclear accident, the explosion at the nuclear power station near Chernobyl, Ukraine. To date, most public concern has centred on the short-term effects of the disaster, especially on the immediate health consequences for people who lived or worked in the area around the failed reactor.

The papers by Baker *et al.*¹ (page 707 of this issue) and Dubrova *et al.*² (page 683) are, however, likely to redirect attention to the long-term genetic and environmental consequences of the accident. These studies document surprisingly high increases in mutation rates in two species of vole, and humans, exposed in varying degrees to radioactive and other contamination from Chernobyl. The reports will stimulate a reassessment of the genetic and other biological effects of exposure to nuclear waste, which until now have been estimated mostly from laboratory studies^{3,4} and investigations of the long-term consequences of the atomic bombs dropped on Hiroshima and Nagasaki^{5,6}.

Baker *et al.* examined two species of vole that live immediately adjacent to the failed reactor and compared them to control populations of the same species from a relatively unaffected region 32 kilometres to the southeast. The voles at the reactor site live in highly contaminated surroundings and consume highly contaminated food. Individual animals are themselves extremely radioactive and are living and reproducing in the presence of unprecedented levels of radiation, as well as heavy metals and other chemical pollutants.

Baker *et al.* analysed nucleotide sequences of a mitochondrial gene that codes for cytochrome *b*, and they estimated a substitution rate from a phylogenetic analysis of the population data of over 2×10^{-4} substitutions per nucleotide site per generation for both species. This unexpectedly high figure was then confirmed by direct analysis of cytochrome *b* sequences from a female vole and its offspring (the mitochondrial genome is ma-

ternally inherited). These direct data also help rule out the possibility that the extreme variation among individuals at the reactor site results from immigration from surrounding populations.

The study by Dubrova *et al.* indicates that the increases in mutation rate are not

populations are not an ideal control group, because there are many other potential population and environmental differences between Belarus and Britain that are unrelated to the Chernobyl accident. This deficiency was ameliorated to some extent by taking relative exposure among the Belarus populations into account (as measured by caesium-137 surface contamination), which indicates a dosage effect of radiation exposure on observed mutation rate (or a dosage effect of other pollutants correlated with radioactivity).

Both the quantitative and qualitative results from these studies are unexpected⁷. The estimated substitution rates in the exposed voles are at least two orders of magnitude greater than any previously reported for mitochondrial protein-coding genes, and every adult vole examined in the contaminated area differs from the others in its amino-acid sequence for cytochrome *b*. Such high rates of substitution in protein-coding genes have previously been observed only among some RNA viruses. Substitution rates of the order of those estimated have not been considered possible for eukaryotes, because they would result in hundreds of thousands of mutations per generation if the rates extended to the entire genome. But substitution rates in mammalian mitochondrial genes are decoupled from the substitution rates in nuclear genes⁸, and Baker and colleagues' study shows that extremely high rates of change can be sustained in the mammalian mitochondrial genome — at least in the short term.

In both analyses^{1,2}, the observed increase in mutation rates is not fully consistent with the expected consequences of radiation damage, which mostly produces length changes in DNA that result from double-stranded breaks. Even the doubling of observed mutations reported for the human minisatellite data is more than can be explained directly by this mechanism⁹. Moreover, the vole cytochrome *b* sequences show an extreme excess of substitutions, rather than length changes to the gene.

The fact that substitution rates are so

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REASONS

Site of disaster — the shattered remains of reactor 4 at Chernobyl, photographed three days after the explosion.

limited to species living and reproducing in the immediate vicinity of the Chernobyl reactor. Human populations in Belarus, a few hundred kilometres north of the disaster site (see map, page 683), received acute doses of iodine-131, and continue to be exposed to more stable isotopes such as caesium-137 as well as other chemical pollutants from the fallout. Although the levels of environmental contaminants are much lower for these people than for the voles living next to the reactor, length changes in nuclear minisatellite loci are about twice as common in the human population in Belarus as in control populations in Britain. Clearly, the British

Gamma/Frank Spooner Pictures

high in populations near the reactor nonetheless points to some detrimental impact of the accident, even if radioactivity isn't the sole cause. Other explanations for the increased mutation rates seen in both studies include effects from non-radioactive pollutants such as heavy metals, damage to mutation-repair mechanisms, or disruption of physiological processes that secondarily result in higher mutation rates. Whatever the cause, the extreme substitution rates observed in the mitochondrial genome of the voles are highly unlikely to extend to nuclear genes, because the hundreds of thousands of amino-acid replacements that would occur in each generation would almost certainly be fatal.

Despite the high rates of mutation around Chernobyl, many plant and animal populations, including the voles, continue to thrive¹⁰. This is not necessarily inconsistent with the observed increases in mutation rate, as long as the most extreme rates do not extend across the nuclear genome. The reactor site represents an area of reduced competition, reduced predation and abundant food for species such as voles. The increased mutation rate is probably resulting in a reduced lifespan for individual voles, but many are obviously living past the age of first reproduction. Even if local populations experience mutational overload, immigration from surrounding areas may sustain their viability. The long-term consequences of these conditions are unknown and are likely to vary dramatically between species; in general, however, species with short generation times, small genomes and high dispersal rates are the most likely to survive the short term in the most contaminated areas.

The accident at Chernobyl was a great tragedy, but its after-effects provide an unprecedented opportunity to gather information on the genetic effects of nuclear waste. The fallout from Chernobyl is so distinct from that following the bombing of Hiroshima and Nagasaki, both in the extent and diversity of pollutants, that predictions from one to the other are not likely to be very meaningful¹¹. The studies at Chernobyl are therefore providing the

first glimpses of the genetic effects of severe nuclear accidents. Further research will be needed to address the generality of the results, the consequences of increased mutation rates for the viability of the organisms affected, the distribution of mutations across the genome, and the mechanisms responsible for increased mutation rates. The extreme mitochondrial substitution rate in the voles even makes possible the extension of experimental evolutionary studies to eukaryotes (to date such studies have been restricted largely to viruses¹²). In effect, millennia of change are being compressed into a few years. Of course, whether such dramatic increases in mutation rate can be sustained by the populations

concerned is also an open question.

Meantime, the new papers leave little doubt that the genetic consequences of the Chernobyl accident are great and will be long-lasting, even though the causes, mechanisms, distribution, extent and phenotypic effects of the mutations are as yet unknown. We now know that the mutational effects of nuclear accidents can be much greater than suspected, and that evolutionary rates in at least parts of eukaryotic genomes can be raised well beyond levels previously considered possible. □

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TAXONOMY

Birds in double trouble

Graham Martin

WE have come to accept that evolutionary change and the process of speciation in birds can be rapid^{1,2}. But nothing has prepared ornithologists for the possible doubling of recognized bird species now being advocated by some taxonomists and aired at a meeting last month*. A world list of 20,000 species, as opposed to the current 10,000, has become a very real prospect as the techniques of DNA analysis threaten to displace field notebooks as the main tool of avian taxonomy.

For half a century ornithologists have had it relatively easy. Despite vicissitudes of funding, they at least have had the near certainty of knowing what they were studying. Species have appeared to be well defined and, apart from relatively minor and local problems, there has been general agreement around the world concerning species lists, although the evolutionary relationships between them have been much debated³. This certainty about species has facilitated rapid international exchange of information, not only within ornithology but between a wider audience of bird watchers, opinion formers and policy makers.

In consequence, birds have been readily adopted as a focus of conservation concern, and in so doing they have become surrogates for wider ecological issues. Much national and international legislation uses bird species as a simple tool to force ecological issues to the fore. This is exemplified by the controversies in North America over Californian condors and spotted owls, and in Europe over white-headed ducks. This apparent certainty over what constitutes a bird species has, however, been on shaky ground, and over

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

How many three-toed woodpeckers? This bird, *Picoides tridactylus*, has a worldwide distribution ranging from the Alps of Central Europe through Scandinavia and eastwards through northern Russia to the Pacific and throughout the higher latitudes of North America. It is currently regarded as a single species divided into about 12 subspecies⁸. But application of the phylogenetic species concept, employing analysis of mtDNA from individuals taken from four of these subspecies on either side of the Bering Strait, has shown sufficiently large differences in the mtDNA to suggest that these subspecies should each be recognized as a separate species⁷.

the past decade a dispute has surfaced which threatens to polarize opinion and in so doing has implications well beyond the boundaries of scientific ornithology.

The meeting brought together ornithologists to explore the issues and search for common ground. Many delegates must

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* *Avian Taxonomy from Linnaeus to DNA*, London, 23 March 1996.

have left the meeting, however, wondering how far we have moved since 1859, when Darwin commented that "No definition has yet satisfied all naturalists; yet every naturalist knows vaguely what he means when he speaks of a species".

The debate centres on two principal species concepts: the biological species concept (BSC), neatly defined by Mayr⁴ as "Species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups"; and the phylogenetic species concept (PSC), defined by Cracraft⁵ as "A species is the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent".

The difference between these two ideas can be summed up as one between definitions focusing on a process or on a pattern (R. Zink, Univ. Minnesota). The ramifications of this simple dichotomy are, however, fundamental. It was argued that the distinction between pattern and process really hinges on the debate between regarding ornithology as a science dominated by field work or by laboratory investigation (R. Liversidge, McGregor Museum, Kimberley, South Africa). The first requires painstaking observation of what birds actually do in nature, who they choose to mate with and so on, whereas the second is becoming increasingly concerned with the analysis of minute observable morphological traits or patterns of mitochondrial DNA (mtDNA)^{6,7}. Thus the argument can also be seen as one between those who recognize the definition of species in ornithology as based upon an accessible science, in which an amateur armed with binoculars, camera and notebook can still contribute, or a professionalized pursuit where only those with funds and access to laboratories will have the final say.

There is also the question of what ornithologists believe avian taxonomy is for. Is it primarily a tool rather than an end in itself? Should taxonomists be mainly seen as serving 'customers' in the wider scientific and amateur ornithological community (J. Greenwood, British Trust for Ornithology)? These customers need to know how to partition nature to ease the development and communication of explanatory ideas and look to taxonomists to provide these species partitions. Seeing taxonomy and the definition of species as tools was a powerful theme of the meeting, with most of the customers present being happy with BSC, and in no mood to purchase the new-and-improved tool of a PSC if it is to be based upon 'invisible' mtDNA.

The wider implications of adopting the PSC approach were made graphically clear in discussions of the subspecies concept, which is widely used in ornithology to describe diversity (D. Snow and

A. Knox, formerly of the Natural History Museum, London). Adoption of PSC leads inevitably to the loss of the subspecies concept, either through lumping of subspecies together into new species groups or elevating a subspecies to species status. In the case of many currently recognized species which have wide geographical distributions and much diversity of form, the lumping together of subspecies would lead to a loss of recognition of that diversity (Snow). On the other hand, raising many subspecies to species status would result in a plethora of new species, each with a tiny geographical distribution, and perhaps indistinguishable in the field (Knox) (see figure). It was agreed that application of the PSC approach as currently used could double the world list of birds species to about 20,000 — which would be a boon to ornithological publishers but a mixed blessing to 'tickers' who seek ever longer lists of species seen.

More seriously, a doubling of bird species would affect the status of birds in international wildlife conservation. The introduction of a large number of species which are defined by characteristics that are difficult or impossible to detect in the field, and which may also have limited geographical distributions, would make it

impossible to apply much of the standing legislation applying to national and international bird conservation and species protection (N. Collar, Birdlife International, Cambridge). It would change the unit of biodiversity dramatically, and make the policing and monitoring of conservation measures more difficult.

Clearly, this is a controversy that is only just beginning, and the meeting showed it to be one based upon fundamentally different viewpoints. It is not yet time to throw away your trusted field guides, but it could well be time to start saving for the double-sized revised editions. □

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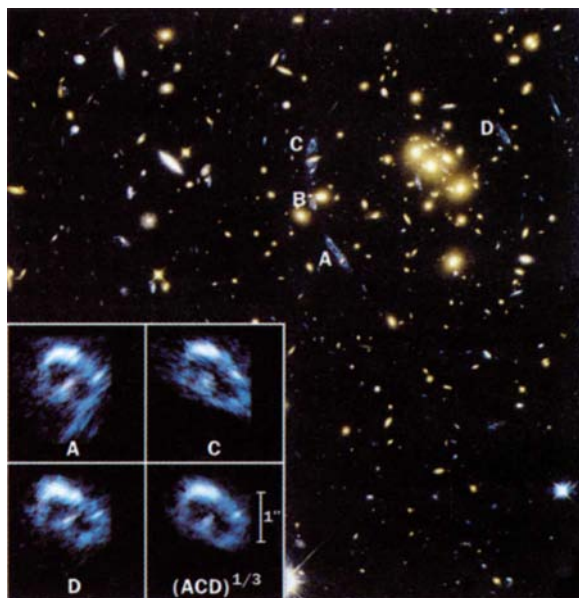
ASTRONOMY

Unique half-eternity ring

THE massive cluster of galaxies shown here acts as a gravitational lens, bending the light from a more distant galaxy so that it appears to us as five blue arcs surrounding the brightest area of the cluster. This Hubble Space Telescope image reveals clear detail in each arc.

W. N. Colley, J. A. Tyson and E. L. Turner (*Astrophysical Journal* **461**, L83–L86; 1996) have traced light rays back through a lens model that includes the overall cluster dark-matter distribution and dozens of individual galaxies. By adjusting the lens parameters they found a model that reproduced the five arcs with a unique source image: the beaded, ring-like galaxy shown in the inset, separately reconstructed from three of the arcs (and all three in combination).

It appears to be a young galaxy in the process of formation. What we see as blue light was emitted as ultraviolet



by hot, massive stars when the Universe was less than half its present age, probably in a 'starburst' phase of galaxy evolution. Similar galaxies are seen closer to us, but this is a rare view of such a distant example. Star formation may only be occurring in a ring, or perhaps the dark patch inside the ring is due to absorption by huge amounts of dust. Stephen Battersby

How the Galaxy keeps its halo

J. Michael Shull

OVER the centuries, our understanding of the Milky Way has undergone a vast evolution, from the substance of mythology, to early astronomical explanations as a diffuse nebula, to the modern view of a rotating disk of stars and gas embedded in a huge Galactic halo. The halo consists of mysterious dark matter (low-mass stars or exotic particles) together with considerable amounts of visible gas. Forming a cocoon around the Milky Way's disk, the halo stabilizes the disk gravitationally, stores its cosmic rays and acts as a reservoir for the stellar and gaseous debris blown out of the disk. Connections between the disk and halo are therefore of great interest to astronomers. On page 687 of this issue¹, Normandeau, Taylor and Dewdney make a compelling case for the discovery of a 'Galactic chimney', a conduit for replenishing halo gas from the disk. Using the 21-cm emission line of atomic hydrogen as a tracer, they have mapped a cone-shaped cavity of gas flowing out of the disk above a group of massive stars in the Perseus spiral arm.

The term chimney was coined² to describe plumes of hot interstellar gas formed above associations of massive (O- and B-type) stars in the disks of spiral galaxies. The interstellar medium in galaxies is a complex system, regulated in part by the life and death cycles of massive stars. As these stars evolve over their brief (3- to 10-million-year) lives, they shed mass in high-velocity winds before dying in violent supernova explosions. Because most massive stars form in clusters and associations, their stellar winds and supernovae are concentrated into small regions of space and brief intervals of time, magnifying their impact on the surrounding gas. The resulting large cavities of hot gas ('superbubbles') are surrounded by denser, compressed layers ('supershells' and chimney walls) that have been pushed back by the cavity pressure. Riding on the plume of hot gas is a region of turbulent, cooling gas. In theoretical models³, the most energetic of these superbubbles are capable of breaking out of the gaseous disk, lifting the material great distances into the halo (Fig. 1).

The region of the Milky Way explored by Normandeau *et al.* lies near a Galactic longitude of 135 degrees, about 2,200 parsecs (pc) from the Earth. The Perseus chimney is 120 pc in width and extends 110 pc above the star cluster, and includes

nine O-type stars in the cluster OC1352. The cone-shaped outflow sits directly above a region of ionized hydrogen gas (Fig. 2). The winds from these nine stars have a combined kinetic luminosity of about $3 \times 10^{37} \text{ erg s}^{-1}$, or about 30 per cent of the $10^{38} \text{ erg s}^{-1}$ of a typical OB association. Equally interesting are observations of 'streamers' of H I (neutral



FIG. 1 False-colour radio image of the Perseus chimney. The chimney is the dark, roughly cone-shaped area surrounded by luminous gas, which is ionized by the nine bright O-type stars at the base of the chimney, marked by crosses. The image is roughly 150 parsecs across.

hydrogen), possibly representing flows of gas evaporated from two molecular clouds within the cavity. The velocities across the cavity range over about 50 km s^{-1} , suggesting the beginnings of a plume.

The Perseus chimney is probably just one of many disk-halo conduits scattered throughout the Galaxy. Radio observations of the nearby spiral galaxy M31 have found hundreds of holes in the H I distribution⁴, thought to be where superbubbles have punched through the gas layer. In the Milky Way, radio surveys have also uncovered a number of large shells⁵ and vertical structures called 'worms'⁶, which could be of superbubble origin. In Cygnus, Orion and other active star-forming regions, X-ray telescopes have found large cavities of hot interstellar gas. Even the local bubble⁷ of hot gas within 100–200 pc of the Sun is thought to be a fossil superbubble from an old cluster of O and B stars.

Several questions are raised by these observations. How many superbubbles

exist across the Milky Way disk? How many of these break through the disk and into the halo? How far into the halo can these plumes rise? Only the most luminous OB associations are likely to produce enough energy to form chimneys that break out of the disk, and such associations are rare. To make a quantitative estimate, assume a type-II-supernova rate of one every 40 years, spread throughout a Galactic disk of radius 15 kpc, with 50 per cent of the supernovae occurring in clusters of 100 massive-star progenitors (each of which will produce a supernova). If each such cluster produces a chimney that stagnates at a radius of 100 pc after 10^7 years, then in a steady state there would be more than 1,000 chimneys, occupying about 6 per cent of the area of the disk. The mean distance between chimneys would be around 750 pc. But few of them are likely to penetrate the halo, and by considering only the most energetic OB associations one arrives at a much smaller number of large chimneys, perhaps only 100.

One can reverse the question and ask how many chimneys are required to replenish the Galactic halo. However, this question is poorly defined, as the sizes and energies of superbubbles cover a broad range, from 10^{51} to 10^{54} ergs in energy and 100 to 1,000 pc in size. The intermediate-temperature halo gas seen in emission and absorption by triply-ionized carbon requires about $10^{40.6} \text{ erg s}^{-1}$ of power to replenish its radiative losses, setting a lower limit on the total hot gas supply to the halo. Estimates of the power needed to supply all the updraughts of hot gas and the infall of cool clouds, together known as the 'Galactic fountain'⁸, range from 10^{40} to $10^{42} \text{ erg s}^{-1}$, with a gas input of 1 to 10 solar masses per year. The upper range of this power requirement corresponds to a Galactic supernova rate of one (10^{51} erg) supernova every 30 years, at the upper limit of what is inferred from pulsar birth rates. But the power injected into chimneys by the stellar winds of O-type and Wolf-Rayet stars rivals that of supernovae. During the critical early stages of a chimney's lifetime⁹ the energy injection by OB associations is dominated by these winds. It would be no surprise if the early stages of chimneys and plumes are controlled by stellar winds and not by supernovae.

The Perseus chimney needs considerably more study before we understand the dynamics and thermodynamics of the outflow. Can we see X-rays from the hot cavity? Is the cavity capped by a swept-up gas shell, or is the plume open to the halo? As new observations determine whether the outflow has enough velocity and pressure to break through the disk's H I layer, an analysis of the size and shape of chimneys may decide whether ionizing photons from O-type stars are a major source of

Medial temporal lobe imaging

James V. Haxby

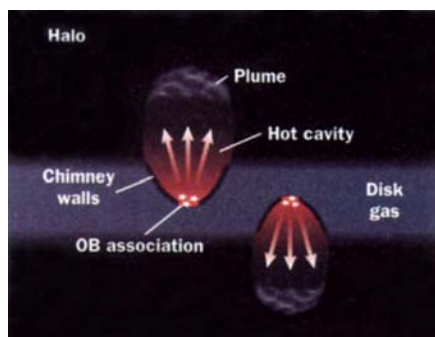


FIG. 2 Schematic view of part of the disk and halo of the Milky Way. Two chimneys are shown (there would probably be more like 100 in total), their plumes supplying the halo with hot gas. The young hot stars at their bases may also be responsible for photoionizing the halo gas.

the diffuse hydrogen line emission¹⁰ in the lower halo. By fracturing the disk's H I absorbing layer, chimneys may serve as conduits for this ionizing radiation as well as for hot gas¹¹.

On the grander scale of the intergalactic medium, galactic halo studies are needed for the proper interpretation of quasar absorption lines. If these absorbers arise from intervening galaxies, with 50- to 100-kpc haloes contaminated by heavy elements from supernovae, then it is tempting to attribute the halo matter to plumes blown out by superbubbles from the disk. But can chimneys really rise as high as 50 kpc, or could the heavy elements instead be the result of supernovae in the halo? Understanding chimney penetration into the halo will be a critical factor in this debate. □

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Correction

Credit for the electron micrograph of foamy virus accompanying Robin Weiss's article of 21 March (*Nature* **380**, 201; 1996) was misattributed. The picture was provided by Dr Hans Gelderblom of the Robert Koch Institute, Berlin.

ALTHOUGH it has been clear for four decades that the brain's medial temporal lobes play a critical role in memory processing^{1,2}, functional brain imaging has not yet managed to produce consistent evidence for an association between medial temporal lobe activity and verbal memory^{3–5}. This is surprising, given that imaging methods are now able to provide reliable and sensitive measures of activity in separate medial temporal lobe structures. But far from eroding the validity of the association with memory, this inconsistency highlights the need for innovation in imaging research methods and a better understanding of memory processing before we can clarify the contribution of medial temporal structures in establishing and retrieving memories.

A report by Nyberg *et al.* on page 715 of this issue⁶ represents a major advance in this endeavour. It provides the first unambiguous demonstration that the medial temporal lobe is active at the time of verbal memory retrieval. Moreover, the success of this study is due in large part to advances in experimental design and data analysis. Innovations in these methods can be as critical to progress in human brain mapping as improvements in imaging technology.

Certainty about memory function in the medial temporal lobe comes from studies of amnesia. Bilateral lesions in the medial temporal lobes, due to infection, stroke, hypoxia or surgery, can result in an almost total inability to commit new information to long-term memory, with no impairment of intelligence or language. Recall of memories learned before the brain injury can also be impaired.

Uncertainty remains, however, regarding the mnemonic roles played by different medial temporal structures, such as the hippocampus, the parahippocampal gyrus and the rhinal cortices. The basis of the memory impairment in amnesia is unknown. It could be due to disruption of medial temporal participation in encoding, in retrieval, in the consolidation or stabilization of memories over time, or in all of these processes. By demonstrating that medial temporal activity is associated with the act of retrieval, the results of Nyberg *et al.* indicate that brain structures in this region do participate directly in retrieval.

The experimental design of most imaging research is based on the subtraction method — activity during a baseline task is subtracted from activity during an activation task. In a well designed experiment, the activation and baseline tasks differ on only one cognitive operation of interest. Early imaging studies of memory

chose tasks that were assumed to differ in the extent to which they engaged long-term memory operations on the basis of neuropsychological studies of amnesia or cognitive psychology studies of memory performance.

For example, amnesics can perform normally on tests of short-term memory (subspan) but are impaired on tests of recall for longer lists (supraspan), indicating a specific impairment of long-term memory. Imaging studies contrasting supraspan and subspan learning of lists, however, did not demonstrate any change in medial temporal activity^{7,8}. In an example from cognitive psychology, instructions that direct attention to the meaning of a word rather than to its sound or physical features result in better recall. Imaging studies contrasting encoding the meaning to encoding the physical features of words, however, have not demonstrated any change in medial temporal activity⁴.

These studies do not invalidate the role of the medial temporal lobe in encoding long-term verbal memories. It is possible that the baseline tasks in these verbal encoding experiments also engage the medial temporal lobe⁹. Selection of appropriate baseline tasks is as important as selection of appropriate activation tasks, and the search for baseline tasks that do not elicit medial temporal activity may also provide new insights into memory function.

Tasks that do not require an intact medial temporal lobe for normal performance, such as short-term memory tasks, apparently elicit medial temporal activity in the normal brain^{7,8,10}, presumably to form a new long-term memory even though the task can be mediated by a short-term memory process. Tasks that result in poor long-term recall, such as encoding the physical features of words, may also elicit medial temporal activity, but the long-term memory that presumably results from this activity is a representation of features that do not facilitate retrieval. Three imaging studies of verbal memory encoding, which will be presented at upcoming meetings, do appear to have used

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Charles Oatley (1904–96)

PROFESSOR Sir Charles Oatley died in Cambridge, England, on 11 March. His death marks the end of a long chapter in the history of the scanning electron microscope (SEM) — arguably the single most important scientific instrument to emerge since the Second World War. The first 17 years of the development of the SEM in its modern form took place in his laboratory at Cambridge, and led directly to its industrial production and wide application.

Following a higher education in physics, and a period in industry and at King's College London, Oatley spent the war years at the Radar Research and Development Establishment, Malvern. In 1945 he was elected a fellow of Trinity College and appointed to a lectureship in the engineering department at the University of Cambridge. His remit was to modernize the teaching of electronics, which was then called 'electric signalling', and to set up a research group. He wrote that one of his criteria in the choice of a research project was that "it should be adventurous and speculative"; electron optics was to be included but he did not want to trespass on the ground of V. E. Cosslett who was setting up a transmission electron microscopy (TEM) group in the Cavendish Laboratory.

Oatley thought that the SEM would be a suitable project, although attempts during the 1930s by Knoll and von Ardenne in Germany, and by Zworykin in the United States, to develop it for the direct imaging of surfaces, had been largely abortive. The experts he consulted were all opposed to the scanning concept, but he nonetheless decided to assign a research student to work on it: "in the worst case it would lead to a PhD and the failure to achieve the desired result would not be a disaster". I was lucky enough to be the student selected (in 1948), probably because I had had some years' experience in radar and the television industry.

The first rather rudimentary SEM worked in 1951 and from the start produced the striking three-dimensional

images of surfaces that are now commonplace. One reason for this success was that the metal specimen was etched and placed at an angle to the scanning beam, and the contrast was topographic. The earlier workers had intended to use variations in the secondary emission ratio to produce contrast, a forlorn hope in the typical vacuum of an electron microscope (although Knoll was successful because



he used baked and sealed-off vacuum tubes for his experiments). It is only comparatively recently that microscopists have become aware that an ultra-high vacuum microscope is essential for meaningful secondary electron imaging.

Oatley realized that with further development the SEM could be a most useful instrument. At that time, surface structures were imaged indirectly in the TEM by means of replica techniques which could achieve high resolution. The comparatively low resolution of the early SEMs was one of the reasons why electron microscopists were indifferent to the many advantages of direct imaging of surfaces; another was that Oatley's group was outside the mainstream of electron microscopy and "anyway they were only gifted amateurs".

Undeterred, Oatley continued work on the project with a succession of research students, in particular K. C. A.

Smith, who greatly improved the first SEM and produced images comparable with those from modern instruments; even so, it was many years before the virtues of the SEM came to be generally recognized. Meanwhile Oatley's aim remained: to have the SEM marketed. Finally, after one fruitless attempt by a leading electron microscope manufacturer, in 1965 the Cambridge Instrument Co. Ltd announced the world's first production SEM, the Stereoscan.

The SEM has since been applied in virtually every scientific field, and instrumental developments continued: high-resolution (0.5 nm), low-voltage operation for non-conducting specimens, and the environmental SEM for wet specimens. Electron-beam microfabrication, which was first demonstrated in Oatley's group in 1964 by A. N. Broers, has had a major impact in the manufacture of microchips.

Oatley became professor of electrical engineering in 1960, and was knighted in 1974. He had retired three years previous to that, but continued with the development of the SEM, including carrying out single-handed a meticulous investigation on electron detectors; he read a paper on this topic at the symposium held in honour of his eightieth birthday. He then concentrated on tending his beautiful garden that had been a lifelong interest.

All of his students regarded him with great respect and affection, and many have had outstanding careers. To name but three, I. M. Ross became president of Bell Telephone Laboratories and T. E. Everhart of the California Institute of Technology, and A. N. Broers is professor of electrical engineering and vice-chancellor elect of Cambridge University.

Charles Oatley is survived by his wife, Enid, and his sons, John and Michael.

Dennis McMullan

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baseline tasks that do not elicit spontaneous left medial temporal activity (J. F. Binder, L. Nyberg and J. V. Pardo, personal communications). All demonstrate increased left medial temporal activity as compared to tasks that divert attention from linguistic features of stimuli.

Additionally, more sensitive analyses of medial temporal activity can be designed that do not rely on the simple subtraction method. The study by Nyberg *et al.*⁶ effectively uses two alternative approaches. Subtraction did not reveal significant

modulation of medial temporal activity in their study. Instead, correlations between regional blood flow during a single task and behavioural performance were used to reveal the association of medial temporal activity with retrieval success. These authors also introduced a new multivariate method for image analysis, partial least squares, which was able to demonstrate changes in medial temporal activity between tasks that univariate contrasts between tasks did not demonstrate.

Amnesia provides the basis for one of

the most solid links between brain and behaviour — the link between the medial temporal lobe and memory. Perhaps future imaging studies will help unravel the relative contributions of the different medial temporal lobe structures to the encoding, consolidation and retrieval of memories. □

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Shocking states of matter

Russell J. Hemley and Neil W. Ashcroft

HYDROGEN is not only the most abundant element in the cosmos, it is also a benchmark system for the study of condensed matter physics. It is just a single electron bound to a single proton, but this simplicity is deceptive when many atoms are brought together. In the familiar gas form, hydrogen is a diatomic molecule with a potent covalent bond. At very high pressures, however, the consensus has been that electrons will eventually be stripped, severing the bonds and forming a dense state with mobile, conducting electrons. At high temperatures and pressures, such a state in fluid form is thought to constitute the bulk of the giant planets. But like the solid form of dense hydrogen, it has remained largely the subject of theoretical fancy here on Earth. A recent paper¹ and a corresponding announcement at the March Meeting of the American Physical Society has changed this state of affairs. A group at Lawrence Livermore National Laboratory (LLNL) reports the metallization of a fluid state of hydrogen under shock compression.

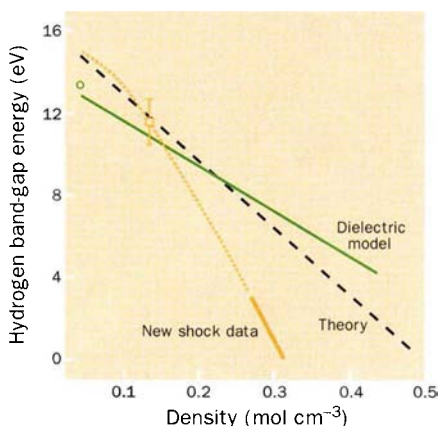
One of the early triumphs of quantum mechanics was to predict how matter can exist, and in what forms, at the very high densities found in certain astrophysical bodies. Fowler² showed that hydrogen atoms could collapse under extremes of pressure and temperature to form a dense state, the plasma state, favoured because its electron kinetic energy is lower than in the gas. That a dense conducting state could also form at low temperatures as a solid was later predicted by Wigner and Huntington³ in a study that launched a steady stream of theoretical investigations, including predictions that the crystalline metal may exhibit exotic physical properties such as very high-temperature superconductivity⁴.

Effort has mounted to study both the high-temperature fluid and low-temperature solid by, respectively, dynamic and static compression techniques. The road has been a difficult one because of the extreme pressures and/or temperatures required. But in the past few years, advances in both experimental areas have given insight into this ubiquitous material in both its high-density forms.

The recent advance comes from shock-wave physics. In a dynamic compression experiment, pressure and temperature increase together. The highest pressures are achieved at a shock front propagating through the sample. The problem in conventional shock-wave studies of hydrogen has been that the temperature often climbs too quickly relative to the pressure. For example, work done several years ago by the LLNL group⁵ showed that in a

single shock in hydrogen the temperature reached more than 3,000 K with the pressure increasing to the relatively low value of 10 GPa (100,000 atmospheres), far below that expected for the formation of a conducting state.

Electrical probes did show that the material became modestly conducting at these pressures, but it occurred by excitation of electrons across the gap between valence and conduction bands, a gap that diminished as the pressure increased. Moreover, the experiments provided a measure of the pressure- (or density-)



The band gap for hydrogen as a function of density — a band gap of zero means that at least some of the electrons in the substance are free to conduct, and it has become a metal. The yellow line is the fit to the high-temperature shock-wave data of Weir *et al.*¹ for the fluid. The square is the earlier shock-wave result⁵. The circle and green line are from dielectric model fits to diamond-anvil cell optical data, which track the direct band gap for the low-temperature solid⁷. The dashed line is a theoretical calculation for a model of the rotationally disordered solid at zero kelvin. The use of more recent pressure-density data⁷ will shift the new shock results to higher densities.

induced decline of the band gap, gratifyingly close both to the best theoretical calculations⁶ and to optical data for the low-temperature solid⁷. The same theoretical calculations and experimental data on the band gap clearly indicated that higher pressures were needed to reach a conducting state, the most likely route being closure of the band gap⁸, which can be facilitated by dissociation.

High-pressure research is ever pushing the extremes. In the past two years, the LLNL group has developed a new technique in which a shock wave bounces back and forth off the walls of the cell that holds the hydrogen sample, and 'rings up' the pressure to higher and higher values before the cell is destroyed. Moreover, calculations indicate that it does so without

the problematic increase in temperature.

The conductivity of fluid hydrogen increases sharply with pressure in these new experiments. At lower pressures, as with the earlier LLNL work, this could still be understood in terms of thermal excitation of electrons across a band gap. But at pressures above about 1.4 million atmospheres a very clear change in the trend was observed. As Weir *et al.* now report¹, a plateau in the dependence of conductivity on pressure was reached at 140 GPa and 3,000 K — compelling evidence for a degenerate, fully conducting state. The measured conductivity at high pressure falls in a range which is comparable to that of other metallic fluids, but significantly smaller than that expected for the fully dissociated hydrogen plasma. Just as the dense hydrogen in Jupiter is veiled beneath its cloud tops, so too some properties of a material in a shock experiment cannot be observed directly but must be modelled. The magnitude of the conductivity is consistent with just a small degree of dissociation, the models of the LLNL group indicating a value of about 5 per cent. Evidently, a metal forms through the closure of a band gap (see figure), and with most of the hydrogen pairs remaining intact, a picture far from the fully dissociated, and originally envisaged, dense plasma².

Notably, the same calculations that suggest both high-temperature superconductivity⁴ and band overlap⁸ in the solid metal also point to the possible high stability of H_2^+ in dense phases, the ionized form proposed by Weir and colleagues¹. However, it is important to note that even at the 5-per-cent level the dissociated protons would also contribute significantly to the conductivity through other changes in electronic structure. Also, the pressure-volume state calculated in the LLNL model differs from that expected from lower-temperature data⁷, and that will shift the reported onset of high conductivity to higher densities (see figure).

As hydrogen is notoriously difficult to contain at high pressures (it is very reactive) and these are the first experiments of their kind, the results are viewed with a degree of caution. It is encouraging that they appear to confirm an early magnetic compression study of hydrogen⁹, but there is an important difference. The formation of a metallic fluid was claimed at 200 GPa and 400 K, but Weir *et al.* attribute the conductivity in that study to thermal excitation across the band gap.

Perhaps the most interesting question raised is the relationship between the reported high-temperature metallic fluid and the low-temperature solid. Static experiments show a striking number of new phenomena in the solid form in this very pressure range (~150 GPa), including a surprisingly complex phase diagram, remarkably strong infrared absorption bands and evidence of bond weakening⁷. But

papers published a week before^{10,11} the new data reported essentially no optical signature of metallization in the solid at low temperatures and comparable pressures.

Whether that presents a problem for the high-temperature liquid, or whether it points to the crucial role played by temperature when the gap is known to be getting small, remains to be seen. It is important to note that the phase of a substance can strongly affect its electrical properties — for example, at room temperature and atmospheric pressure, crystalline silicon is a semiconductor, but at high temperature it melts to form a liquid metal. Calculations show that solid hydrogen adroitly finds its way to lower energy states and avoids metallization by continually re-orienting its molecules as pressure is increased. In these states unusual quantum effects enter into the determination of hydrogen's physical properties. There may also be important temperature effects on the band gap, again something well known in ordinary semiconductors. The high temperature of the shocked hydrogen produces a distribution of intermolecular distance that includes close encounters between molecules; these will modify the charge distribution, which in turn is reflected in the band gap.

There are implications for planetology and fundamental physics. One of the remarkable features of Jupiter is its intense magnetic field, presumably associated with the convection of metallic hydrogen deep in the jovian interior. The LLNL measurements allow the possibility of a conducting fluid forming at lower pressures, so the magnetic field may be generated at shallower depths than previously thought. Moreover, the new results suggest that the boundary between the insulating, molecular and the metallic, non-molecular layers is gradual and continuous, rather than sharp as had been assumed.

The authors comment that their observations also rule out the existence of a theoretically predicted electronic phase transition within the fluid at higher temperatures and pressures — the so-called plasma phase transition¹². Yet the

existence of just such a transition is indicated by recent highly accurate quantum simulations¹³, though these are restricted to lower densities. Even more intriguingly, the boundary for the plasma phase transition from that work, when extrapolated to lower temperatures and pressures, is remarkably close to the metallization point reported by Weir and colleagues. In general terms, the distinctions between the state reached experimentally and the conventional classical plasma are not sharp. All of this hints at a possible and previously unsuspected consistency among recent results, from low to very high temperatures.

ECOLOGY

Body size and biodiversity

Sean Nee and John H. Lawton

It is easy to be both enchanted, and bewildered, by the diversity of life on Earth. An obvious reaction is to concentrate upon the particular, as natural historians have done for generations. But doing good natural history is only one component of doing good science. Ecologists need to discover the patterns and rules that underpin the seemingly endless lists of species¹.

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Natural History Museum, London

So, according to the LLNL experiment, fluid metallic hydrogen finally makes a fleeting appearance. But far from closing a chapter in the study of hydrogen, the result poses many new questions about the dense states of the most abundant element in our Universe. □

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very easy to be overwhelmed by so many bugs! Instead, from this daunting pile emerge striking but hitherto unnoticed basic patterns linking species richness, relative abundances and body size. (For the same reasons that journalists report people's ages, ecologists record body size: no single number is so informative⁵ and it is easily measured.)

The most striking visual pattern to emerge is that the number of species within each body-size class, S , the total number of individuals in that class, I , and body size (calculated as 'biovolume') form a parabola when viewed in three dimensions (see Fig. 1d on page 705). Projected onto the SI plane to see how the number of species scales with the number of individuals, a power-law relationship emerges. S is proportional to $I^{0.5}$: that is, a 10-fold increase in the number of individuals within a size class generates, on average, 3.2 times more species. Moreover, this relationship holds across a 100,000-fold range of insect body sizes, and five different orders. Underpinning this remarkable result is an apparently even deeper pattern, one that has not hitherto been explored by ecologists, to which we now turn.

A classic area of enquiry in community ecology is the pattern of relative abundances between species, which is bound up intimately with our notions of 'diversity'. If the species in a community do not differ greatly from each other in abundance, then the community exhibits greater diversity than if, say, one species is numerically dominant. If we rank the species in a community according to their relative abundance, from commonest to rarest, then the way in which abundance declines with rank is one representation of the abundance distribution. Siemann and colleagues ask another new macroecological question: what is the relationship between abundance, A , and rank, r , within

Insects — basic patterns link species richness, relative abundances and body size.

Insects at one and the same time present a particular challenge and advantage — they are the most speciose class of organisms on Earth, dominating terrestrial ecosystems; but it is relatively straightforward, albeit labour intensive, to sample entire assemblages.

Both the challenge and the advantages are beautifully illustrated by Siemann *et al.* on page 704 of this issue². Working in the grasslands and savannahs of Cedar Creek in Minnesota (the site is rapidly becoming one of ecology's classic localities^{3,4}), they collected insects by sweep-netting throughout the 1992 field season. This simple but effective technique, which is exactly what it sounds like, yielded 89,596 individual insects assigned to 1,167 species in five main orders. It would be

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size classes? The answer is once again a power law: A is proportional to $r^{-1.94}$.

They show elsewhere that, with a few assumptions that are not frightening, this relationship within size classes will generate the overall power-law relation between numbers of species and numbers of individuals. So if we possessed a theoretical understanding of how power laws might reasonably arise in this context, then Siemann and colleagues would be in a position to offer a pre-cooked explanation of both discoveries. However, power-law rank–abundance relations are largely neglected in the canonical literature on the subject^{6,7}, which is otherwise well equipped with models. Two previous studies of insects also found such a relationship across their entire sample^{8,9}. But these were studies of tropical insect communities, and the greater diversity of the tropics, combined with much smaller sample sizes, means that it is difficult to know whether one is observing a genuine power-law distribution, or simply the tail of a distribution, such as a log-normal, that can mimic a power law. The present study gives greater confidence that there is some worthwhile theorizing to be done, because existing models for power law rank–abundance relations in other areas of biology¹⁰ do not have an obvious relevance in this context.

Unusually for studies of insects, we have considerable confidence in this case that the sample is large enough to reveal true community patterns. Within each body-size class, by taking ever-larger subsamples of the data, it is possible to construct so-called species-accumulation curves, which show how the numbers of species in the sample increase with sample size. Here, for almost all body-size classes, the accumulation curves appear to be levelling off towards an asymptote, which would be the true number of species in the community. The results are robust when the analyses are repeated using estimated, asymptotic, species richness rather than the observed numbers of species.

Leaving aside the striking patterns re-

vealed by this pioneering study, there are at least two further messages to be drawn from it. Our guesstimates of the total number of species on Earth received a shocking upward revision from studies of tropical insects¹¹. Siemann and colleagues present a sanguine view that their study may incline us to the lower end of the 10–50 million range of estimates. But the only body-size class that did not exhibit an asymptote in their species accumulation curve was the smallest, which means that it is impossible to develop a reasonable statistical estimate of the total numbers of small insect species, even in this enormous study in the temperate zone.

Second, the evidence presented in the paper² makes another macroecological observation more robust. It is known for many groups of animal — birds, mammals

and fish — that the distribution of body sizes is skewed, so there are more relatively small species than large ones^{12,13}. This right skewness is also evident in the five orders of insects studied here (Fig. 2a, page 705), but there is no obvious reason why this should be the case.

Sweep-netting insects in a field may not look like hard science. But this remarkable study shows that bug hunting produces more than just endless lists of species, and poses some hard problems for theoreticians to muse over. □

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CLIMATE VARIATION

Cycling around the South Pole

Xiaojun Yuan, Mark A. Cane and Douglas G. Martinson

MANY of the variations in climate we all observe (and enjoy, or suffer) seem to be randomly distributed in time and space. While this view has an irreducible element of truth, there is increasing evidence that much of the Earth's climate variability arises from a structured, global, interconnected system. On page 699 of this issue¹, White and Peterson provide a striking example, with evidence for an interannual 'Antarctic Circumpolar Wave' (ACW). This is not a water wave but a disturbance in sea-ice extent, in sea surface temperature, in surface wind speed, and in atmospheric pressure at sea level. Although the short history of measurements in the Antarctic region rules out a definitive description, the data available from the past 15 years show a two-wavelength ACW propagating around Antarctica (see Fig. 1) with a frequency of about 4–5 years.

This wave in the ocean, atmosphere and cryosphere (ice) is a strong and significant part of the interannual variability in the southern polar region.

Interannual variability in the Antarctic region may affect the global climate by altering the Equator-to-pole temperature gradients which drive the dynamics of the atmosphere and oceans. A more likely influence is the unique ability of the Antarctic Circumpolar Current (ACC) to transport oceanic anomalies between the three major oceans, Pacific, Atlantic and Indian. Variability in the Antarctic may influence the global climate on longer timescales by changing formation rates of deep and bottom waters, and thus, ultimately, the heat transfer between ocean and atmosphere. By identifying and characterizing an important mode of variability in the Antarctic region, the discovery of the

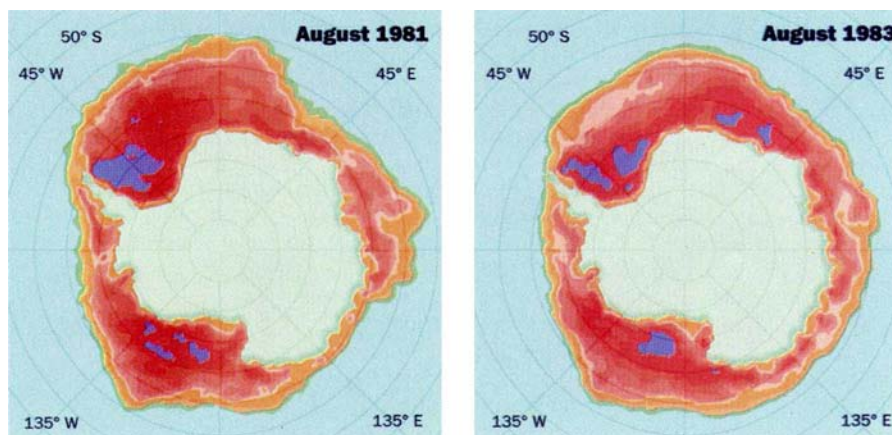


FIG. 1 Mean Antarctic ice concentration, August 1981 (left) and August 1983 (right). The shape of the ice coverage is very different between years, and there are two detectable bulges that move around with the Antarctic Circumpolar Wave. (Taken from images made by the Scanning Multichannel Microwave Radiometer satellite. Image courtesy of NASA.)

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FIG. 2 Half a metre of snow, resting on sea ice that comes just one inch above the water. The picture was taken in the Weddell Sea in May 1992, while new ice was forming in the open water. Local observations of sea ice missed the continent-wide ACW.

ACW opens new directions for research on the interannual variability of the global ocean-atmosphere-cryosphere system.

What causes the interannual variation in the Antarctic region? White and Peterson speculate that it may be ENSO signals propagating to high latitudes through the atmosphere. ENSO, an acronym for El Niño/Southern Oscillation, originates in the equatorial Pacific, and is the best known mode of interannual variation in global climate², one which has been shown to have a substantial impact on human affairs^{3,4} (causing droughts and floods, forest fires, crop failures and the collapse of fisheries). Numerous studies have associated ENSO events with climate anomalies in middle latitudes as well as the tropics. With this latest addition we can say that ENSO may dominate interannual climate variability even in high latitudes.

Prior studies^{5,6} had pointed to ENSO signals in Arctic and Antarctic sea-ice fields. However, the particular Antarctic signal examined (total ice coverage, for example) often did not fully register the ACW sea-ice mode and so underestimated the strength of the connection. Observations of Antarctic sea ice over smaller areas or shorter times⁷⁻⁹ did reveal some cyclical components in the interannual variability, but their incomplete view of the polar region concealed what we now see to be the passage of a quasi-periodic wave. The ACW framework suggests a way to fit these pieces of information into a coherent pattern, though it remains to be seen just how well they will fit, and how the completed pattern will look.

We note that correlations between anomalies in the Antarctic ice edge and standard indices of ENSO (sea surface temperature in the eastern Pacific and sea-level pressure difference between Tahiti and Darwin — see for example ref. 4) imply that 40 per cent of the variance in the sea-ice anomalies is attributable to ENSO. The Antarctic sea-ice anomalies

Ted Baker

have even higher correlations with precipitation over tropical land areas (28° N to 28° S, 85.5° W to 152.5° E) and with sea surface temperature in the equatorial Indian Ocean (2.5° N to 12.5° S, 62.5° to 82.5° E). The correlations are not significantly higher than the one with ENSO, however, and both of these measures are themselves strongly related to ENSO. So they raise the possibility that the sea-ice variation may not be entirely determined by an ENSO signal emanating from the Pacific, but do not settle the issue. Regardless, there is strong evidence connecting the

ACW to tropical climate variations.

Even with the knowledge that tropical signals propagate to higher latitudes through both ocean and atmosphere, it can hardly be said that the physics of this connection is understood. White and Peterson¹ point out that the propagation speed of the ACW is the same in all three media, which supports the quite plausible idea that the atmosphere, ocean and cryosphere are strongly coupled on interannual timescales. Indeed, that the ACW stands out in the data for all three is *prima facie* evidence of such interactions. They also note that its speed falls within the range of speeds seen in the ACC, and go on to suggest that advection of sea-surface-temperature anomalies produces the wave, with the time for a complete circumpolar circuit setting the period at 4–5 years. But it is not clear how such a mechanism maintains synchronization with ENSO. Furthermore, the speeds within the ACC are too variable to pick a unique period.

In any event, by discerning such an elegant structure amid the disorder of the climate data, White and Peterson offer us an intriguing puzzle with global climate implications. □

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DAEDALUS

Fuse and lose!

WHY is obesity so common? One theory is that mothers overfeed their babies, to maintain their recommended increase of weight. The fat cells in the infants' bodies multiply rapidly to store all this nutriment. The extra cells persist for the rest of life, eager to seize and store whatever fat comes their way. Only surgery can get rid of them.

Daedalus now has another way: cell fusion. Cells in a culture can easily be induced to fuse together. Specific chemicals, deactivated viruses, or pulses of direct or alternating high voltage can all briefly disrupt the membranes of two cells in contact, causing them to fuse. The resulting joint cell can be perfectly viable. Indeed, fused cells are often found in samples of human tissue, and seem to cause no harm.

But how to work the trick inside the body? Alternating-current fusion probably works by inducing ultrasonic agitation in the cell membrane, which then resonantly captures electrical energy. So Daedalus is combining two well-known medical devices — the diathermy machine, which launches alternating current into a large volume of the body from two contoured electrodes; and the ultrasound scanner, which focuses a beam of ultrasound in a tiny volume of tissue. Cunningly, he will slave them together, to work at exactly the same phase and frequency: 500 kHz or so. The tissue at the ultrasound focus, resonantly excited at that frequency, will then absorb much of the electrical power of the diathermy machine. Most of the cells in that region will fuse. By scanning the ultrasound focus through a whole volume of fatty tissue, most of its cells could be fused. At a stroke, the patient will have half the number of fat cells, each of twice the normal size.

Now a big cell is inefficient; it can't service its large volume through its inadequate surface. The fused fat cells will be forced to shrink, which they will do by flipping into 'discharge' mode, and discarding their excess fat. The patient will lose weight and volume dramatically. When the fused cells have all returned to their previous size he (or she) will be carrying just half the previous burden of fat. If necessary, the process could then be repeated.

Fatties everywhere will rush for the treatment. A reduced fat-cell count will solve their problems permanently. Even better, controlled scanning could remove fat from exactly the regions causing distress, without deflating other more becoming volumes. Forget dieting, ignore exercise and let Daedalus's electro-ultrasonic body sculpture shape the figure of your dreams!

David Jones

Phylogenesis of prion protein

SIR — We have reconstructed the phylogeny for various mammal species based on the sequence of the prion protein gene. By tracing the pattern of amino-acid substitutions through the phylogeny, we have identified two pairs of derived substitutions uniquely shared by cattle (*Bos taurus*) and the hominoids. Although we have no specific explanation for these events, a phylogenetic analysis can help to indicate their significance. Two factors make the result compelling: the probability that it occurred by chance is estimated to be less than 1.2×10^{-4} ; and the substitutions, although of a conservative nature, occur in a region of the gene postulated to be involved in the acquisition of prion diseases.

The prion protein is constitutively expressed in adult vertebrates and bound to the surface of neurons¹. Its function is unknown, but in modified form it is associated with several neurodegen-

erative diseases, including kuru, scrapie², Creutzfeldt-Jakob disease (CJD)³ and bovine spongiform encephalopathy (BSE)⁴. Many point substitutions have been identified which either lead to disease directly or modify susceptibility to disease through latency effects⁵. The evidence suggests, therefore, that the gene contains many sites of large effect.

The phylogeny is shown in the figure; the coloured circles indicate the most likely positions in the tree where the amino-acid substitutions occurred. The ancestor of the hominoids (excluding the orangutan (*Pongo*)) and of *B. taurus* uniquely shared a tyrosine-to-histidine replacement at site 155, and an asparagine-to-serine replacement at site 143. The probability of two correlated pairs of binary events occurring on this tree was estimated by a maximum-likelihood procedure⁶ to be less than 0.005. The null model states that all amino-acid replacements at the

sites are equally likely. The asparagine-to-serine replacement requires one nucleotide substitution, yielding six amino acids that could have occurred. The tyrosine-to-histidine replacement similarly requires one nucleotide substitution, yielding seven possible amino acids. Thus, the probability of observing the particular pair of amino-acid replacements at these sites, given that they were observed once elsewhere in the tree, is less than $0.005 \times [1/(7 \times 6)] = 1.2 \times 10^{-4}$.

The number of similar pairs of amino-acid replacements expected on the phylogeny by chance can be estimated by considering all possible similar pairs of sites along the gene. Fifty-six positions on the gene show at least one evolutionary amino-acid replacement in the phylogeny, seven show two, and four show three. If the 56 sites are taken as those showing any evidence of change, we obtain 1,540 possible different pairs of sites that might have shown the effect we observed. The expected number of correlated pairs of transitions such as we observed is thus $1,540 \times 1.2 \times 10^{-4} =$

0.18 (95% confidence interval = 0–0.55). Basing this calculation on the 11 sites that changed twice or more yields a figure of 0.0065 (95% confidence interval = 0–0.019). The event we observe is the only one of its kind in the phylogenetic tree.

Given the improbability that the convergence we describe occurred by chance, we can reflect on its biological significance. One possibility is that the substitutions confer resistance of the cellular prion protein to modification to the pathogenic form of the prion protein. Alternatively, they may improve the efficiency of this protein in the healthy tissue of these species. As an incidental consequence of the latter, these changes might also have predisposed humans towards a strain of prion disease occurring in *B. taurus*. Substitutions of a conservative biochemical nature have previously been implicated in disease symptoms in other studies of spongiform encephalopathies¹. Mutations in the amino-acid residues between sites 90 and 145 have been shown to correlate with the onset of disease⁷. Of the two substitutions we report, one falls within and the other is adjacent to this region.

The transmission of sheep-derived scrapie to humans has never been proved, despite the existence of endemic scrapie for more than 200 years⁸. The transmission of BSE to humans from cattle has recently been suggested⁹ to result in a previously unrecognized form of CJD with a novel neuropathology, although there is no direct evidence for this proposal. Although statistically minded scientists are ever mindful of Hume's dictum that correlation is not causation, we should nevertheless remain attentive to rare events that might be associated with the emergence of what may be a new strain of this disease.

David C. Krakauer

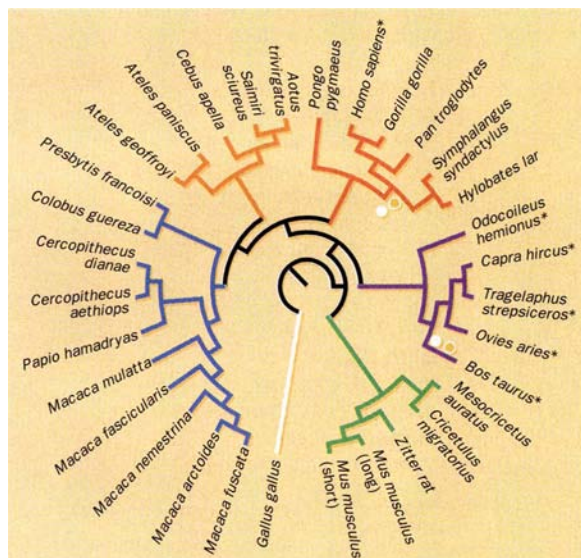
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Phylogenetic relationships among the prion protein genes of 33 species of vertebrates estimated from a 750-nucleotide sequence using maximum-likelihood methods¹⁰. Higher taxa are colour-coded according to the following key: red (hominoids), purple (artiodactyls), green (rodents), blue (Old World monkeys), orange (New World monkeys), and aves in white used as an outgroup. The two reconstructed amino-acid substitutions are marked with circles. The white circle marks the Tyr-to-His (155) substitution, and the yellow circle the Asp-to-Ser (143) substitution. Of interest is that the gene tree topology is inconsistent with the species tree topology. For example, the Old World and New World monkeys are sister groups in the gene tree, whereas the Old World monkeys and the hominoids are sister taxa in the species tree. *Homo* is a sister group of *Gorilla* in the gene tree, whereas in the species tree, *Homo* is the sister species of *Pan*. Short- and long-latency mouse variants of a single species are also shown. The tree is a strict consensus of 100 DNAML maximum-likelihood trees, assuming a Kimura two-parameter model of evolution with a transition-transversion parameter of 2.0. Asterisks identify species known to contract spongiform encephalopathies naturally.

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Age of a millisecond binary pulsar

SIR — The recently reported properties of the millisecond binary pulsar J1012+5307 and its white-dwarf companion¹ suggest that the ages of such systems can be substantially overestimated. Lorimer *et al.*¹ determined a characteristic age of the radio pulsar to be 7,000 million years (7 Gyr), whereas they estimated the companion to be at the most 0.3 Gyr old. The latter age was based on cooling timescales for white dwarfs, as calculated by Iben and Tutukov², and led to the suggestion that the pulsar did not start on the spin-up line, but near its current spin period of 5 milliseconds^{3,4}. Our evolutionary calculations indicate, however, that the cooling timescales of very-low-mass white dwarfs (LMWDs) of mass $M_{\text{wd}} \leq 0.25 M_{\odot}$ can be considerably underestimated by the traditional white-dwarf cooling curves, which were deduced for $M_{\text{wd}} > 0.3 M_{\odot}$.

We calculated the formation and cooling of LMWDs in binary systems using a recent version of the evolution code developed by Eggleton⁵. The equation of state is extensively described by Pols *et al.*⁶. Our calculations indicate that the cooling timescale of a LMWD can be substantially larger than described in ref. 2 (see figure), because we saw no thermal

flashes that resulted from thermally unstable shell-burning, as reported in other work^{2,7,8}. This was the case even if the time step in our calculations was as small as 50–100 years.

Our results appear to confirm Webbink's⁸ finding that white dwarfs with $M_{\text{wd}} < 0.20 M_{\odot}$ do not show thermal flashes. The main effect of thermal flashes would be the reduced hydrogen content in the envelope. Because of the high luminosities reached, hydrogen is rapidly burned and may be lost via Roche lobe overflow. In Iben and Tutukov's model² (with $M_{\text{wd}} = 0.30 M_{\odot}$), there is still an amount of hydrogen $M_{\text{H}} = 1.6 \times 10^{-3} M_{\odot}$ in the envelope when the white dwarf reaches its maximum effective temperature. This is comparable to our results. In Iben and Tutukov's model, two thermal flashes with consequential Roche lobe overflow later occur, reducing the amount of hydrogen to $M_{\text{H}} = 0.14 \times 10^{-3} M_{\odot}$. Because thermal flashes did not occur in our LMWD models, the amount of hydrogen in the envelope remained high and resulted in a much longer phase with significant hydrogen burning. This results in considerably longer cooling times of LMWDs that have hydrogen

envelopes: the so-called DA white dwarfs.

The age τ_{wd} of the DA white-dwarf companion of PSR J1012+5307 is then defined as the time from the moment the mass-loss from the progenitor of the white dwarf stops until the effective temperature has decreased to the present value $T_{\text{eff}} = 8,481$ K. This last value, and the surface gravity $\log g = 6.5$, have been determined by fitting optical spectra — which indeed show strong Balmer lines of hydrogen — with theoretical models (M. H. van Kerkwijk, P. Bergeron and S. R. Kulkarni, manuscript in preparation). For a LMWD cooled down to $T_{\text{eff}} = 8,481$ K, our calculations show that τ_{wd} can be as high as approximately 8 Gyr (see figure), depending on the mass of the white dwarf (for a given composition). Assuming $\tau_{\text{wd}} = \tau_{\text{pulsar}} = 7$ Gyr, we find: M_{wd} approximately $0.185 M_{\odot}$, $\log g$ approximately 6.7 and P_{orb} approximately 1 day. These are close to the measured values $\log g = 6.5$ and $P_{\text{orb}} = 0.6$ day. The final period in our calculations could be smaller if one assumes a little more efficient magnetic braking.

We thus conclude that all observational and theoretical evidence is consistent with an age of the white-dwarf companion of PSR J1012+5307 of the order of 7 Gyr, which agrees with the characteristic age of the pulsar.

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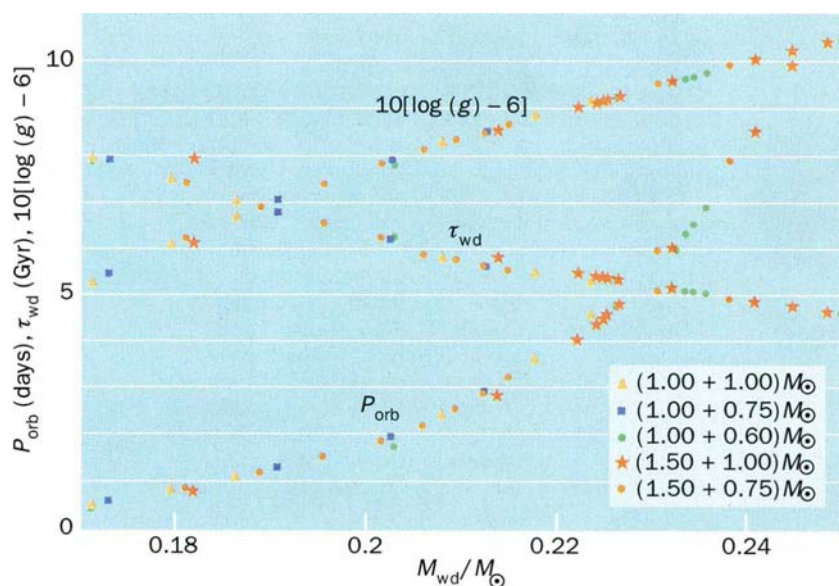
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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. Priority will be given to letters of fewer than 500 words and five references. Manuscripts can be submitted to London or Washington.



The orbital period P_{orb} , $\log g$ and the age τ_{wd} plotted as a function of mass M_{wd} for a white dwarf which has cooled down to $T_{\text{eff}} = 8,481$ K. We assume that the progenitor of the white dwarf is initially a zero-age main-sequence (ZAMS) star ($X = 0.70$, $Z = 0.02$) which fills its Roche lobe at a certain moment on or after the main sequence, losing mass to a compact object of unspecified origin. When almost the entire envelope is lost, the hydrogen envelope collapses and the contact star becomes a detached helium white dwarf. The mass of the white dwarf depends on the moment when the mass transfer in the system starts and is for short orbital periods in the range 0.17 – $0.25 M_{\odot}$. The lower mass limit is determined by the bifurcation between converging and diverging systems⁹. Results are shown for binary stellar systems with different initial conditions. Each symbol denotes a specific set of initial masses, written in the key with the ZAMS star first and then the compact object in parentheses.

Role of leptin in fat regulation

SIR—Leptin administration reduces body weight and adipose tissue mass. However, the weight loss observed cannot be completely attributed to decreased food intake^{1–3}. In rodents, brown adipose tissue is the primary thermogenic organ, and it has become increasingly clear that brown adipose tissue is significant in regulating body composition. Brown adipose tissue can be activated by acclimation to cold temperatures and by excess food intake. These effects appear to be mediated through noradrenergic stimulation^{4–6}. Indeed, early studies demonstrated defective thermogenesis in genetically obese (*ob/ob*) and diabetic (*db/db*) mice^{7,8}.

Recently, we have shown that leptin seems to be a sensor of adipocyte mass, and that treatment with a thermogenic β_3 -adrenergic receptor agonist reduces both adipose tissue mass and leptin expression⁹. Therefore, we hypothesized that leptin's role in regulating fat mass might include signalling the sympathetic nervous system to increase thermogenesis and energy expenditure in brown adipose tissue as fat mass increases. In this case, white adipose tissue mass would be regulated by brown adipose tissue activity through leptin-stimulated sympathetic outflow.

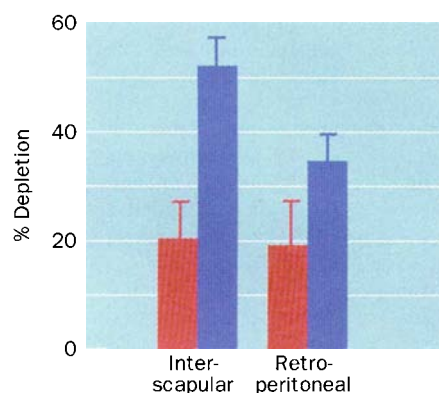


FIG. 1 Effect of leptin on the rate of noradrenaline depletion in brown and white adipose tissue depots after synthesis inhibition with AMPT. Groups of 12 mice were injected i.p. with either 100 μ l phosphate-buffered saline (PBS; red columns) or 100 μ l PBS containing 40 μ g recombinant leptin (blue columns). Data shown are the average of two independent experiments. Per cent depletion was calculated relative to the time 0 value. Results are expressed as mean $\pm$ s.e.m. for each group. Results were analysed by 3-way ANOVA (fat pad $\times$ treatment $\times$ time), followed by a 2-way ANOVA on specific fat pads (treatment $\times$ time) and *post-hoc* Fisher's protected LSD using SuperANOVA; $n = 12$ mice per group. Asterisk indicates $P < 0.04$.

We determined sympathetic outflow by assessing noradrenaline turnover after blockade of catecholamine synthesis with the tyrosine hydroxylase inhibitor α -methyl-*p*-tyrosine (AMPT)^{10–12}. Twenty-four female C57BL/6J (*ob/ob*) mice at 5–6 weeks of age were given either 40 μ g recombinant murine leptin prepared as described² or saline by intraperitoneal injection 2 hours after the start of the dark cycle (lights off at 1600 h). The mice were fasted for the 12 hours of the light cycle before the experiment, and then each received a weighed amount of mouse chow. Food intake was measured for 2 hours after leptin or saline administration. Animals were then given an intraperitoneal injection of AMPT (250 mg per kg body weight). Half the animals (12 per treatment group) were killed immediately and the second half 4 hours later. These time points were based on previous studies establishing the linearity of the fall in noradrenaline content in murine tissues after inhibition of synthesis¹². Interscapular brown and retroperitoneal white adipose tissue was collected for catecholamine analysis.

Leptin administration resulted in a selective increase in noradrenaline turnover to interscapular brown adipose tissue, but did not significantly affect noradrenaline turnover in retroperitoneal white adipose tissue (Fig. 1). There were no differences in food intake between the groups during the 2-hour period after leptin injection (Fig. 2). This effect of leptin on noradrenaline release is consistent with our hypothesis that thermogenesis in brown adipose tissue may be an important mechanism by which leptin regulates body composition, and could be responsible for the increased metabolic rate and core temperature reported after chronic leptin administration^{1–3}.

Indeed, it is possible that the development of obesity in a transgenic mouse with decreased brown adipose tissue, described by Lowell and colleagues⁵, may be related to the inability of leptin to signal thermogenesis in this system. Because many forms of obesity have been associated with increased leptin levels^{13–15}, it has been argued that 'leptin resistance' may be involved in obesity syndromes. Another possibility is that

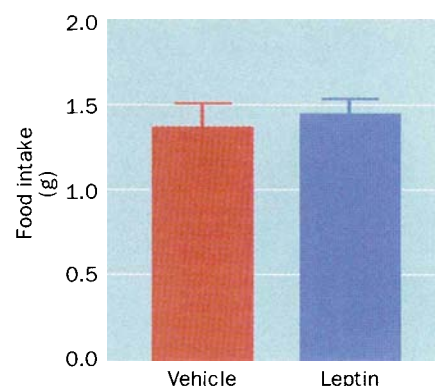


FIG. 2 Food intake in the 2-h period after i.p. injection of leptin or PBS vehicle. The effects of leptin administration are compared with those of vehicle (PBS). Food intake was measured for individual mice during the 2-h treatment period before AMPT injection and analysis of catecholamine levels. Data shown are mean $\pm$ s.e.m. for each group.

some obesity results from the failure of brown adipocytes to respond appropriately to leptin-induced sympathetic activity. In at least one model of diet-induced obesity, brown adipocytes failed to respond adequately to sympathetic stimuli¹⁶.

In summary, we detected increased sympathetic outflow in response to leptin without changes in food intake during a 2-hour treatment period. Therefore, enhanced energy utilization as well as decreased food intake together may account for the weight-reducing effects of leptin.

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Turing patterns in fish skin?

SIR — Kondo and Asai¹ interpret observations on the time evolution of skin patterns of the angelfish (*Pomacanthus*) as the first instance of a Turing (reaction–diffusion) pattern in biology. But we believe that reaction–diffusion systems *per se* cannot provide a mechanistic basis for one of the main patterns reported in ref. 1.

Reaction–diffusion systems are characterized by an intrinsic spatial wavelength of the self-organized concentration pattern, that is, the distance between adjacent peaks of chemical concentrations is determined solely by the system parameters (kinetic constants and diffusion coefficients). Although on a two-dimensional domain such as the fish skin, several equidistant geometrical arrangements of the concentration peaks are possible, the nonlinear terms of the reaction dynamics

usually select only one of these possibilities — for the system chosen by Kondo and Asai, a regular array of stripes. These two features, an intrinsic wavelength and a strong tendency to form stripes, are the essential ingredients of the simulations they presented in ref. 1. Many pattern-forming systems other than reaction–diffusion are known which select an intrinsic spatial wavelength and pattern geometry², among them biologically relevant mechanisms involving chemotactic or haptotactic cell movement and mechanical forces³. Therefore, there is no justification for equating observed patterns with a particular mechanism, as suggested in ref. 1.

Although our point does not exclude the possibility that a Turing system underlies the *Pomacanthus* skin patterns, we demonstrate here that its properties are not sufficient to explain perhaps the most

striking observation of the paper, the regular insertion of new stripes between older ones during the growth of *Pomacanthus semicirculatus*. We have solved the authors' reaction–diffusion equations on a growing, two-dimensional domain — a more realistic representation of the fish skin than the one-dimensional domain used in ref. 1.

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KONDO AND ASAI REPLY — With respect to Höfer and Maini's first criticism, we agree that many pattern-forming systems can explain the phenomenon we observed. These models have in common a set of interactions involving local activation/lateral inhibition coupled with the appropriate nonlinearities⁵. The most important message of our report¹ is that a dynamical mechanism like Turing's is viable for the fish patterns. It should therefore be possible to identify the real molecular mechanism by experiments. Of course, at present the details of the fish-patterning mechanism are unknown, and will not be understood until experiments are done.

Second, Höfer and Maini claim that a two-dimensional simulation of the *P. semicirculatus* pattern is more realistic than the one-dimensional simulation in our paper. This is by no means clear. All the stripe lines of *P. semicirculatus* are perpendicular to the body axis and there are no branch points. These features suggest the presence of a directional preference forcing the stripes to run in the same direction. A one-dimensional simulation captures some of the character of this system better than does an isotropic two-dimensional simulation.

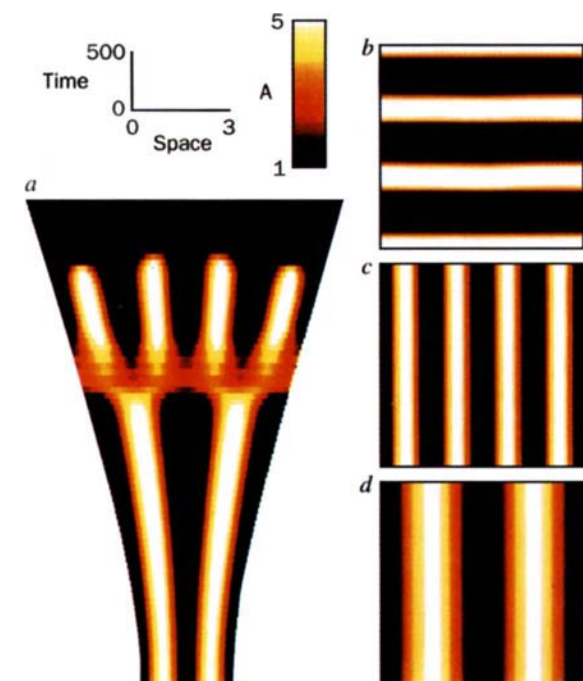
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Behaviour of the Turing system proposed in ref. 1 on a growing square domain (with the signs of the diffusion terms corrected). *a*, Concentration plot of *A* in a horizontal cross-section of the domain; time increases from bottom to top (see separate scales). The 4-stripe pattern produced by the first period-doubling is unstable, rearranges into a 3-stripe pattern perpendicular to the original pattern, and the stripe contours terminate. *b–d*, Snapshots of the stripe patterns corresponding to *a* (domains scaled to same size): *b*, initial 2-stripe pattern ($t = 500$); *c*, after period-doubling ($t = 3,000$); and *d*, after rearrangement into 3 stripes ($2 + 2$ half-stripes, $t = 4,000$, corresponding to the dark region in *a*). Simulations: equations scaled to the form $\partial u/\partial t = s^2 f(u) + D \nabla^2 u$, and solved with a standard ADI scheme on a fixed domain (mesh size 0.2, time step 0.05) with zero flux boundary conditions; increase in s is equivalent to increase in (domain length)², here $s(t) = \sqrt{(0.15 + 10^{-7}t^2)}$. Patterning sequence is sensitive to the speed of domain growth and for faster growth rates the transitions become less controlled; we found transitions from 2 stripes to higher modes (5 stripes and more) with subsequent rearrangements.

striking observation of the paper, the regular insertion of new stripes between older ones during the growth of *Pomacanthus semicirculatus*. We have solved the authors' reaction–diffusion equations on a growing, two-dimensional domain — a more realistic representation of the fish skin than the one-dimensional domain used in ref. 1.

Our results show that regular stripe-doubling sensitively depends on the artificial geometrical constraints of the one-dimensional domain (see figure). As the restriction of one-dimensionality is removed, complete spatial rearrangement of the pattern occurs on the growing domain, which clearly is not seen in the fish. This behaviour is readily explained by the two properties of Turing systems emphasized above. As the domain grows bigger, new stripes should be added, one at a time, approximately conserving the spatial wavelength. Initially, the preexisting pattern appears to force a different sequence of stripe additions to occur, corresponding to the 'period-doubling' behaviour sometimes seen in one-dimensional systems³. However, this situation turns out to be unstable, and the whole pattern rearranges perpendicularly to the old one to

Fields of influence

Paul M. Grant

Driving Force: The Natural Magic of Magnets. By James D. Livingston. Harvard University Press: 1996. Pp. 311. \$24.95, £15.95.

A wonder of such nature I experienced as a child of 4 or 5 years, when my father showed me a compass. That this needle behaved in such a determined way did not at all fit into the nature of events which could find a place in the unconscious world of concepts (effects connected with "direct" touch). I can still remember — or at least believe I can remember — that this experience made a deep and lasting impression upon me. Something deeply hidden had to be behind things.

WITH this excerpt from Albert Einstein's biography, James Livingston, formerly a staff member at General Electric's Corporate Research and Development Center and now a senior lecturer at the Massachusetts Institute of Technology (MIT), opens his delightful little book. Although not all Einsteins, most five-year-olds are nonetheless remarkably inquisitive. I am fortunate to have one at home, my son Diego Patrick, who has recently carried out his own 'experiments' on bar magnets. For him, the repulsive aspect of magnetism is far more intriguing than attraction, and his attempts to defeat Earnshaw's theorem — which states that an object cannot be stably suspended in space by permanent magnets alone — while erratically chasing one magnet with the other are fascinating to watch. I am sure most of us, as Livingston points out was true for him as well as Einstein, have had Diego's experience as part of our childhood introduction to science. Five is about the right age for beginning to marvel and reflect on 'force-at-a-distance', a phenomenon mysterious and beautiful still to us mature physicists — well, to this one certainly.

Driving Force is, in a sense, two books in one. The author attempts to integrate a sociologically oriented exposition of magnetism with an elementary introduction to its science, an interesting and gen-

erally exceptional approach, pioneered some years ago by the popular liberal arts college textbook *Physics for Poets* by Robert March (4th edn, McGraw Hill, 1996) How successful he is depends, I think, on the background of the reader. Personally, I find the lecture-form digressions into technical details distracting, but then, as a practising solid-state physicist, I've already been there. On the other hand, I consider myself a serious lay student of the history and culture of both science and pseudoscience, and it was this side of the book that kept me up late at night. Livingston treats it with a winning combination of dry humour, wit and cheekiness, occasionally spiced with mildly bawdy allusions. It must be a lot of fun to take one of his courses at MIT. (To the best of my knowledge, I have not yet met the author personally, but I am acquainted with some of his former colleagues at General Electric whose stories about him are clearly reflected in his writing style.)

The tone of the book is immediately conveyed by the chapter titles in the table of contents: "Romancing the Stones", "Magnus Magnes", "Thanks for the Memories", "Source of the Force" — all connect magnetism to both past and present human culture and its underlying technology. This connection ranges over an incredible breadth of topics and personalities, a few (nonscientist) examples of the latter being James Bond, Plato, Ben Jonson, Gilbert and Sullivan, the other William Gilbert (Queen Elizabeth's magnetic physician, a scientist of course), Jonathan Swift, Mary Baker Eddy, Dick Tracy, Uri Geller and

Madonna. In addition to a review of the usual and expected applications of magnetism, one finds such exotica as cat doors, continental drift, bird migration, a marvellous variety of magnetic toys, Giambattista della Porta's fantasy telegraph, the non-effect of low-frequency magnetic fields in inducing cancer, magnets and magicians, and magnetism in medicine (some sheer quackery and others developments with great promise, such as magnetocardiography and magnetoencephalography).

Topically, *Driving Force* is pretty much as up to date as could be expected on the



"The magnet and the churn" illustrating a song about unrequited magnetic attraction from Gilbert and Sullivan's comic opera *Patience* (1881). Illustration attributed to William Gilbert.

technical side, including a discussion of giant magneto-resistance and high-temperature superconductivity, and most important historical developments are more than adequately covered. But having been 'present at the creation', I would have liked to have seen more than one paragraph given to the development of magnetic core memories and their enormous influence on the development of digital computers. Also, I looked in vain for clarification of the famous legend that Hans Christian Oersted indeed discovered the existence of electromagnetism while delivering a public lecture. Then again, I enjoyed vicariously re-experiencing the no-holds-barred conflict between Edison on one side and Tesla and Westinghouse on the other over the respective merits of direct versus alternating current in the early days of the electricity industry. Supporters of Edison coined the term 'westinghoused' to describe electrocution by alternating current, surely cause for a defamation-of-character lawsuit in today's litigious society. But with high-temperature superconductors and cryosilicon-controlled rectifiers, Edison might just win in the

ROBOTMAN by Jim Meddick



long run, as low-voltage direct-current transmission and distribution take over in emerging industrial regions globally. Here, however, Livingston makes a cogent observation on materials and technologies in general: "a better figure of merit than energy product is *energy product per dollar*". Harvard, he says, did not teach him this as a graduate student, and nor did it me during my time at that illustrious institution. It is still probably the case.

My few criticisms aside, this is a smashing book. I wish I had written it and I want to write one like it some day. I wish something similar had been around when I was in high school. In an era of

unfortunate public distrust and misunderstanding of the scientific method, I wish there were more science books for the general population written with so engaging an approach. Finally, in the spirit in which the author began his book, and I this review, I wish the 'natural magic of magnets' was used more often to introduce science to children in our pre-schools. It isn't, not in my neck of the woods, and it's a shame. As the Yanks say, "There oughta be a law...". □

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Changing music of the spheres

Paul Murdin

Feynman's Lost Lecture: The Motion of Planets Around the Sun. By David L. Goodstein and Judith R. Goodstein. Norton: 1996. Pp. 224. \$35, £16.99. To be published in the UK by Jonathan Cape in July.

RICHARD Feynman, renowned for his sophisticated course of physics lectures at the California Institute of Technology in the early 1960s, also gave guest lectures. Records of only five of them survive. "The Motion of the Planets Around the Sun", the only publishable one, was discovered by Judith Goodstein, the archivist at Caltech, and has now been released on compact disc with notes edited

by the physicist David Goodstein.

The theme of the lecture is Kepler's first law (1609), which states that the orbits of the planets are conic sections or ellipses. In 1684, Isaac Newton explained to Edmund Halley that simple principles in dynamics and a central inverse-square force were sufficient to give planetary orbits as described by Kepler's three empirical laws, including his second (the radius-vector from the Sun to a planet sweeps out equal areas in equal times) and his third (the 'harmonic law' — the period of a planet is proportional to the three-halves power of its mean distance from the Sun). The second law represents the conservation of angular momentum, the third law comes from the inverse-square law and the first law follows from the other two.

Newton's explanation is a virtuoso piece to perform. Like a musical performance transcribed for modern instruments, Feynman's is, however, not quite a repeat of the original tune, and it has an original cadenza, placed, unusually, at the end.

In *Principia Mathematica* (1687), Newton relied heavily on the properties of conic sections. Feynman follows Newton's geometric methods but uses nothing more advanced than congruent triangles. At first he follows Newton exactly, but then he diverges. "I found I couldn't follow [Newton's proof] very well, because it involves so many properties of conic sections. So I cooked up

another one", he says. Feynman's cooking, presumably independently, follows a recipe published by James Clerk Maxwell, who in turn attributed it to Sir William Hamilton.

Feynman's Lost Lecture goes over some well-trodden history, from Copernicus through Newton to Einstein, and contains reminiscences about Feynman, including the rediscovery of the lecture. There is a transcript of the lecture itself, as well as a compact-disc recording of Feynman delivering it to the Caltech freshman class of 1964. The core of the book is a step-by-step account of his argument that will be accessible to any science student, from sixth-form (high-school) level upwards.

The closing minutes of the lecture display Feynman's gift for lateral thinking. Apparently with time to spare, Feynman turns to Rutherford's experiment demonstrating the small size of the atomic nucleus by alpha-particle scattering. This is a problem of electrostatic repulsion, not of gravity, but the force is a centrally acting inverse-square law and the alpha particles have Keplerian motion. All the Newtonian arguments apply. With them, Feynman derives the probability of the deflection angle of an alpha particle. He uses a treatment by U. and L. Fano from a basic atomic physics textbook of 1959; this seems to have been what gave Feynman the idea of this enjoyable lecture in the first place. □

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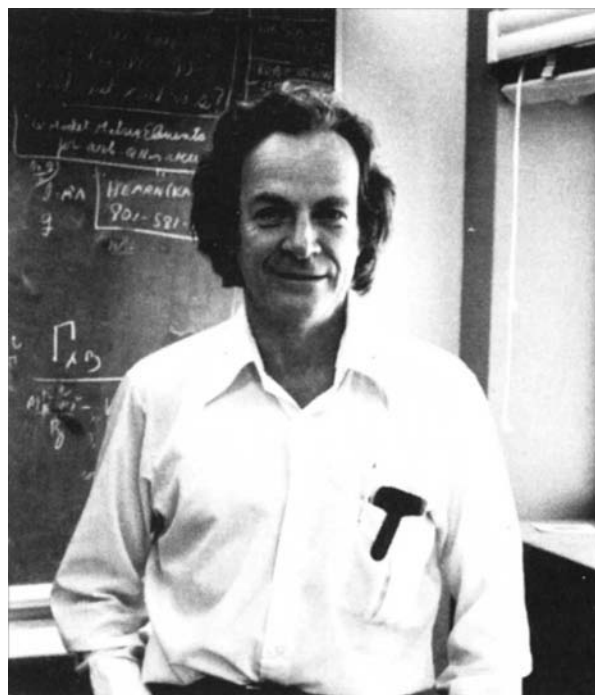
A honeyed society

Ross H. Crozier

The Wisdom of the Hive: The Social Physiology of Honey Bee Colonies. By Thomas D. Seeley. Harvard University Press: 1996. Pp. 295. \$49.95, £31.50.

*That which is not good for the swarm,
neither is it good for the bee.*
Marcus Aurelius

EDWARD O. Wilson, commenting on the work of Karl von Frisch, once wrote that the honeybee is like a magic well to which one can always return for something new and refreshing in the study of social behaviour. The species repays its acolytes by providing all sorts of avenues of investigation not only into selection of a eusocial species (and an economically important and semi-domesticated one at that), but also into the social integration of many individuals to form a smoothly functioning whole. Thomas Seeley stresses the second aspect, providing a largely personal



Feynman's argument will be accessible to any science student from sixth-form level upwards.

reality of the colony as a ground for conflict as well as cooperation makes for complexity, but Seeley inadvertently gives his readers optimism for the future. □

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The ants revisited

Jürgen Heinze

Social Evolution in Ants. By Andrew F. G. Bourke and Nigel R. Franks. Princeton University Press: 1995. Pp. 529. £55, \$75 (hbk); £19.95, \$29.95 (pbk).

The Earth Dwellers: Adventures in the Land of the Ants. By Erich Hoyt. Simon and Schuster: 1996. Pp. 319. \$24.

ANTS grow fungi on chewed-up leaves, tend herds of aphids, engage in territorial wars, and may ruin a picnic almost everywhere. Their fascinating evolutionary success is thought to be largely caused by eusociality: ants live in colonies consisting of one or several reproductive members, the queens, and a much larger number of typically nonreproductive workers. Because of the apparent altruism of the worker caste and the diversity of colony organization, ants have become model organisms in the study of social evolution. A few years ago, Bert Hölldobler and E. O. Wilson delivered a comprehensive account of the behaviour and the evolution of ants in the Pulitzer-prizewinning monograph *The Ants*, praised both for its scientific rigour and for its artistic presentation. One might wonder, therefore, whether there is much room for another ant book.

But *Social Evolution in Ants* by Andrew Bourke and Nigel Franks fills a gap opened earlier this decade. Ant colonies are often believed to function smoothly and without friction among their members. Yet kin-selection theory predicts that the interests of nest-mates eventually diverge. Recent studies have indeed shown that conflicts of interest may strongly shape the pattern of reproduction and the social organization of ant colonies. Bourke and Franks illustrate these and other new aspects of ant life with extraordinary clarity and precision.

Each of the 12 chapters is organized in easy-to-read subunits and ends with a conclusion and a summary. Boxes provide supplementary information about central topics discussed in the text, such as the exact meaning of 'relatedness' and the mechanism of sex determination, or simple calculations to prove central statements. The authors lay much emphasis on a lucid presentation and critical examination of kin-selection



WELL-PRESERVED — The mummy of Nodjmet, wife of Herihor, a member of the Theban ruling family of the Twenty-first Dynasty (around the eleventh century bc). By this time, mummification had been practised in Egypt for at least 1,500 years and had reached the peak of its technical sophistication. *Unwrapping a Mummy* by John H. Taylor. British Museum Press, £9.99.

theory and rival hypotheses for the evolution of social behaviour. In so preparing the way for models devised to explain the origin of 'altruistic' workers and the details of intracolony conflict, they provide a stringent, gene-centred analysis of how selection works, which extends much beyond ants and will be helpful to all wishing to understand social evolution.

Because of haplodiploid sex determination, workers in an ant colony with a single, once-mated queen are more closely related to their sisters than to their brothers, whereas the queen is equally related to both her sons and her daughters. Workers and queens therefore favour different sex-investment ratios. This conflict is additionally complicated by how often the queen mates and the number of queens in a colony. Starting with R. A. Fisher's fundamental model of why equal investment in the sexes is typically evolutionarily stable in diploid organisms, Bourke and Franks elegantly derive sex-ratio theory in ants and provide an excellent overview of recent experimental tests that indicate that workers indeed respond to changed relatedness asymmetries in a colony and manipulate sex investment. Further conflicts arise when workers can produce their own sons from unfertilized eggs or when there are many queens in the same colony. Ritualized or overtly aggressive interactions among nest-mates may then lead to the formation of social hierarchies in which only one or two top-ranking individuals reproduce. The authors review the theory underlying these conflicts and empirical studies, and explain

how ecological parameters and the genetic structure of the colony determine how strongly reproduction is skewed.

Also covered are the fascinating diversity of reproductive strategies, which the authors examine in the light of life-history theory, as well as the division of labour in ants. Here they compare various models such as age polyethism (where division of labour depends on age) with an alternative — or at least supplementary — model, 'foraging for work', where task allocation is more-or-less self-organized.

As a comprehensive review of recent trends in evolutionary biology in ants, *Social Evolution in Ants* is recommended not only to people enthusiastic about social insects but also to those with a broad interest in evolution in general. *The Earth Dwellers* by Erich Hoyt, by contrast, is a mixture of ant stories, scientific information and anecdotes about ant researchers. The naive reader may occasionally find it difficult to distinguish fact from fancy. Do ants really feel fear and hear airborne sound? Or do they really engage in slave-raids? With much of the book based on interviews with Wilson, it reads like a second-hand account of information that can be found at first-hand in Wilson's autobiography *The Naturalist* (Island/Viking, 1994) and Hölldobler and Wilson's *Journey to the Ants* (Harvard University Press, 1994). □

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Human minisatellite mutation rate after the Chernobyl accident

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Germline mutation at human minisatellite loci has been studied among children born in heavily polluted areas of the Mogilev district of Belarus after the Chernobyl accident and in a control population. The frequency of mutation was found to be twice as high in the exposed families as in the control group. Mutation rate in the Mogilev families was correlated with the level of caesium-137 surface contamination, consistent with radiation induction of germline mutation.

THE accident on 26 April 1986 at reactor 4 of the Chernobyl nuclear power station resulted in the largest reported accidental release of radioactive material¹. Many regions within the European part of the former Soviet Union were heavily contaminated by radioactive fallout. In the first three months after the accident, acute irradiation of humans occurred through external and internal exposure to iodine-131 with a half-life of 8 days². Following ¹³¹I decay, exposure to more stable isotopes, mainly ¹³⁷Cs, became the main source of radiation risk for people in contaminated regions.

Despite the magnitude of the Chernobyl disaster, little is known about the effects of radiation exposure in human populations inhabiting polluted areas. A considerable increase of thyroid carcinoma in children around Chernobyl has been reported^{3,4}, as well as an elevated frequency of chromosome aberrations in most residents tested⁵. Additionally, the frequency of congenital malformations in newborns and human embryos has increased in heavily contaminated areas of Belarus following the accident^{6,7}. In contrast, nothing is known about the effects of chronic radiation exposure on germline mutation in humans.

Tandem repeat minisatellite loci have several potential advantages for monitoring germline mutations in humans. The very high rate of spontaneous mutation altering allele length (repeat copy number)^{8–10} provides a system capable in principle of detecting induced mutations in relatively small population samples. We have previously shown that acute doses of ionizing γ -radiation cause a significant increase in minisatellite germline mutation rate in mice¹¹, detectable by DNA fingerprint analysis of small numbers of families at doses substantially lower than can be monitored by standard genetic techniques. A similar increase in mutation rate at one unstable minisatellite locus has been found in the progeny of irradiated mice^{12,13}. Here we report that minisatellite mutation rate is also unusually high in exposed human populations following the Chernobyl accident.

Population variability and mutation scoring

Blood samples were collected from 79 families (father, mother, child) inhabiting the heavily polluted rural areas of the Mogilev district of Belarus (Bychovskii, Krasnopol'skii and Cherkovskii regions; Fig. 1). This cohort is composed of children born between February and September 1994 for whom both parents were continuously resident in the Mogilev district from the time of Chernobyl accident. The control sample consists of 105 non-irradiated caucasian families sex matched to the exposed group of offspring. Because the entire Belarus area was contaminated, and it proved logistically difficult to sample Belarus families with children born before the Chernobyl accident, the control group was from the United Kingdom.

DNA fingerprints were produced from all families by using multilocus minisatellite probe 33.15 (ref. 8) and two hypervariable single-locus minisatellite probes MS1 and MS31 (loci *D1S7*, *D7S21*)⁹. In addition, most families were DNA profiled with the minisatellite probes MS32 and CEB1 (loci *D1S8*, *D2S90*)^{9,14}. These probes, chosen for their relatively high mutation rates^{9,10,14}, provided sufficient information to verify the parentage of all children analysed, even in the presence of mutation¹⁰. Mutants were identified as novel DNA fragments present in the offspring that could not be ascribed to either parent. DNA fingerprints were only scored over the well-resolved region (3.5–22 kilobases).

Examples of families with an offspring containing a mutant band are shown in Fig. 2. Using probe 33.15, 18.12 ± 0.36 (s.d. = 5.58) DNA fingerprint bands were scored per offspring in the control group and 17.52 ± 0.35 (s.d. = 3.24) bands in the offspring of irradiated parents ($t = 1.18$, $P > 0.05$; Bartlett test for homogeneity of group variances, $\chi^2 = 0.89$, d.f. = 1, $P > 0.05$). Furthermore, the frequency of band sharing between parents in the control and Mogilev samples was indistinguishable

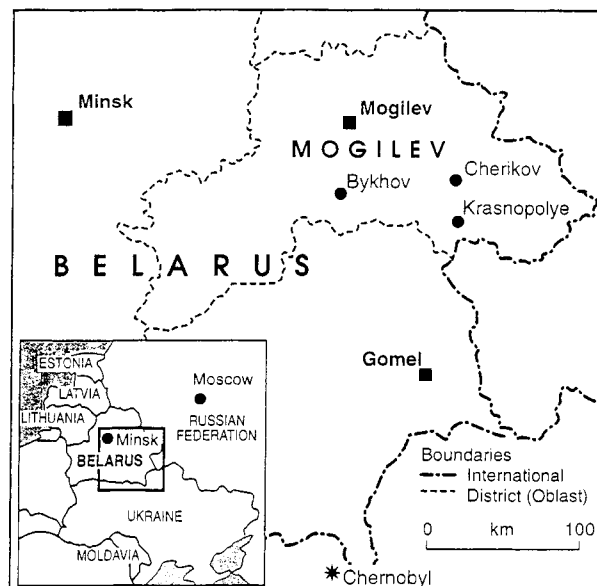


FIG. 1 Map showing the study area.

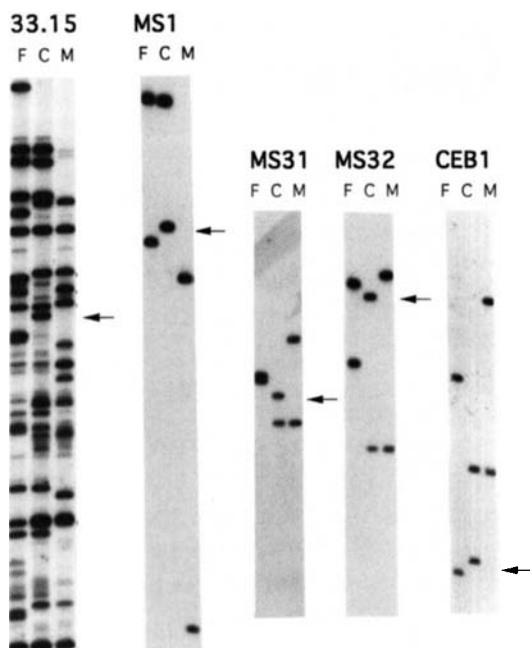


FIG. 2 Examples of minisatellite germline mutation. DNA profiles were produced for each father (F), child (C) and mother (M) using probes 33.15, MS1, MS31, MS32 and CEB1. New mutant bands are arrowed. METHODS. Samples (4 μ g) of DNA extracted from blood were digested to completion with *AluI*, electrophoresed through a 35-cm (for probe 33.15) or 40-cm (for single-locus probes) long agarose gel (SeaKem, type LE, FMC) in $1 \times$ TBE buffer (89 mM Tris-borate, pH 8.3, 2 mM EDTA), transferred to a nylon membrane (Hybond-Nfp, Amersham) and hybridized to 32 P-labelled probes as described elsewhere²⁸. All autoradiographs were scored by eye by three independent assessors. New mutant bands were identified as offspring bands present in neither parent.

(0.124 ± 0.007 and 0.118 ± 0.008 , respectively, $t = 0.57$, $P > 0.05$). Parental allele sizes and allele-length frequency distributions were also determined for the four single loci tested (Fig. 3). For three loci, allele frequency distributions were indistinguishable in the control and irradiated populations; for MS32, minor but significant differences between the two groups were found. We therefore conclude that any differences in minisatellite variability between these two caucasian populations are likely to be negligible.

Mutation rate

Spontaneous mutation rates in the control families were similar to those previously measured in caucasian populations using these five probes^{9,10,14} (Table 1). In contrast, the frequency of mutant bands was elevated in the offspring of irradiated parents. By using multilocus probe 33.15, we found a statistically significant twofold increase in mutation frequency in the offspring of irradiated parents. Reprobing with four single-locus probes showed that some mutant bands detected by probe 33.15 are derived from minisatellites MS1 and MS31, whereas probes MS32 and CEB1 detect sets of bands that do not hybridize with 33.15. We therefore divided mutants scored by 33.15 into those attributable to MS1 plus MS31, and to those from other unknown loci (Table 1). An increased mutation rate in the exposed group was found for both sets of loci. A statistically significant doubling of mutation rate in Mogilev sample was also found at the highly unstable locus *D2S90* (probe CEB1). In contrast, a decrease of mutation rate in the exposed group was found at locus *DIS8* (probe MS32). However, very few mutants were found in either group, and the data do not deviate significantly from those expected for equal mutation rate in both populations (see Table 1) or for a doubled rate in the Mogilev group (Poisson approximation, $P = 0.11$). Finally, we estimated the total frequency of mutant bands in offspring analysed using all three independent probes (33.15, MS32, CEB1); the overall mutation rate was again twofold higher in the Mogilev group.

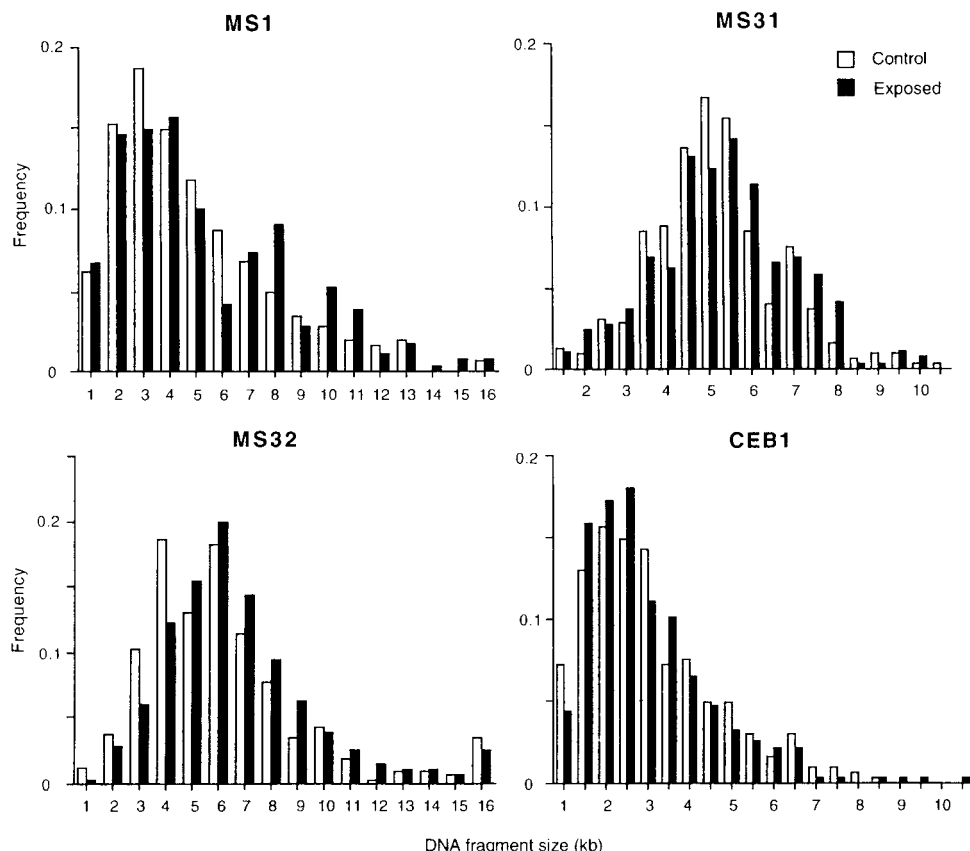


FIG. 3 Distribution of minisatellite allele sizes. DNA fragment sizes were estimated by the method of Southern²⁹, using a 1-kb DNA ladder (GIBCO, BRL) included on all gels, and binned into 0.5-kb (MS31, CEB1) or 1-kb (MS1, MS32) intervals. Differences between the control (white bars) and exposed (black bars) population distributions were analysed using the Kolmogorov-Smirnov two-sample test: MS1, $P > 0.05$; MS31, $P > 0.05$; MS32, $P < 0.05$; CEB1, $P > 0.05$.

TABLE 1 Mutation rates in control and exposed populations

Probe	Locus	Control group			Exposed group			Ratio exposed to control	P*
		Total no. of bands in offspring	No. of mutations	Mutation rate per band	Total no. of bands in offspring	No. of mutations	Mutation rate per band		
33.15:		1,903	19	0.0100	1,384	28	0.0202	2.03	0.0126
-MS1 + MS31†	<i>D1S7, D7S21</i>	387	9 (7 + 2)‡	0.0233	281	11 (7 + 4)‡	0.0392	1.68	0.1794
-other bands		1,516	10	0.0066	1,103	17	0.0154	2.34	0.0288
MS32†	<i>D1S8</i>	164	3	0.0183	150	1	0.0067	0.36	0.5449
CEB1	<i>D2S90</i>	164	11	0.0671	150	20	0.1333	1.99	0.0446
33.15 + MS32 + CEB1§		1,491	23	0.0154	1,615	49	0.0303	1.97	0.0041

* Probability using Fisher's exact test of independence (two-tailed).

† All MS31 and MS32 mutants, including four showing a single repeat unit change, were verified by MVR-PCR analysis of progenitor and mutant alleles^{17,18}.

‡ Number of mutants scored by MS1 and MS31 are shown in brackets.

§ Data presented for the families studied by all three probes.

Blood samples from the control group were collected in the United Kingdom, and it is possible that the increased mutation rate in the Mogilev group might reflect intrinsic differences in minisatellite instability between these two caucasian populations. Indeed, profound allelic differences *in cis* in mutation rate at MS32 have already been found particularly among Africans, where they influence allelic variability¹⁵ yet have little effect on population mutation rate as assayed here. However, increased mutation rate was seen with three groups of independent minisatellites (MS1 + MS31, other bands scored by probe 33.15, and CEB1). Further, very similar increases in mutation rate (1.7–2.3-fold) were found in the exposed group for each of these minisatellite systems, indicating that increased mutation rate cannot be attributed to a single locus that has accumulated unusually unstable alleles in the Mogilev population. We therefore conclude that the difference in mutation rate found between the two groups of families is probably caused by environmental, rather than intrinsic genetic, factors. Environmental mutagens might include industrial or agricultural pollutants as well as post-Chernobyl radioactive contamination.

Surface contamination and mutation rate

Although the exact radiation dose received by each person in the Mogilev sample is not known, the level of surface contamination by ¹³⁷Cs provides a reasonable indicator of collective doses. A significant correlation between the total radioactive fallout after the Chernobyl accident and the level of ¹³⁷Cs contamination was found for most regions of Belarus¹⁶. Families were therefore divided according to median ¹³⁷Cs surface contamination (Fig. 4a) into those inhabiting less contaminated (surface contamination < 6.8 Ci km⁻²) and more contaminated (> 6.8 Ci km⁻²) areas. The total mutation rate (probes 33.15 + CEB1 + MS32) in more-contaminated areas was 1.5 times higher than in less-contaminated areas (0.0390 versus 0.0259 per offspring band, $P = 0.0413$, Fisher's exact test of independence, one-tailed), and both were higher than in the unexposed population (0.0154 per offspring band, $P < 0.05$ for both comparisons; Fig. 4b). The frequency of observed mutations was greater in the more highly exposed families both for 33.15 and for CEB1 + MS32 (data not shown). This correlation of mutation rate within the exposed group with surface contamination levels is consistent with the possibility that the increased frequency of minisatellite mutations found in the exposed group is a direct consequence of irradiation following the Chernobyl accident. It is possible, however, that other non-radioactive contaminants from Chernobyl, such as heavy metals, could be responsible for the observed, apparently dose-dependent increase in mutation rate.

Mutational spectrum

At the four single loci tested, 55 mutants were found, with most mutations at CEB1. The parental origin and germline length

change were defined for each mutant band. The ratio between male and female germline mutation rate is similar in both groups (18 paternal versus 5 maternal mutations in the control group, and 28 paternal versus 4 maternal in the exposed group; $\chi^2 = 0.78$, d.f. = 1, $P > 0.05$). The incidence of mutations involving gain or loss of repeat units is similar in both groups (14 gains versus 9 losses in the control group, and 17 gains versus 15 losses in the offspring of irradiated parents; $\chi^2 = 0.32$, d.f. = 1, $P > 0.05$). Most mutation events involve the gain or loss of only a few repeat units, and the size distributions of mutations are not distinguishable between the two groups (Fig. 5a). The sizes of progenitor alleles are also similar in both groups (Fig. 5b). Mutants at MS31 and MS32 (all paternal) in the control and irradiated families were further characterized by minisatellite variant repeat mapping by polymerase chain reaction (MVR-PCR)^{17,18} to determine the order of variant repeat units along progenitor and mutant alleles (data not shown). Most mutants in both groups showed features expected from previous analyses of mutants identified in pedigrees and by single-sperm analysis¹⁸, namely mutational polarity with changes in repeat copy number restricted to the unstable end of the tandem repeat array, and with evidence in some cases for interallelic transfer of repeats during mutation (data not shown). We therefore conclude that there is no obvious difference in mutation process between the control and exposed groups.

If enhanced mutation is the result of radiation exposure, it appears highly unlikely that minisatellites themselves are the

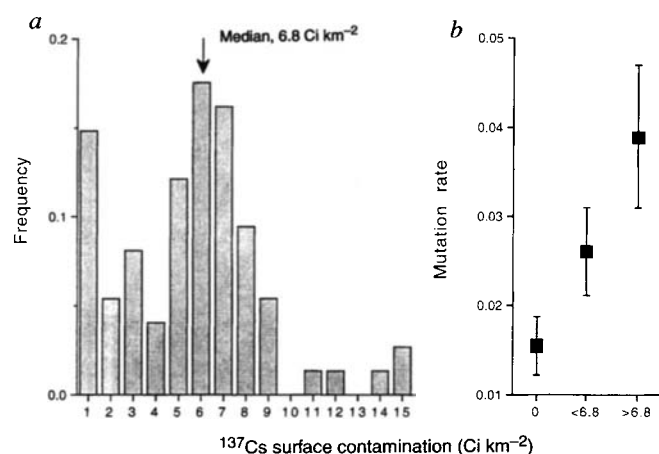
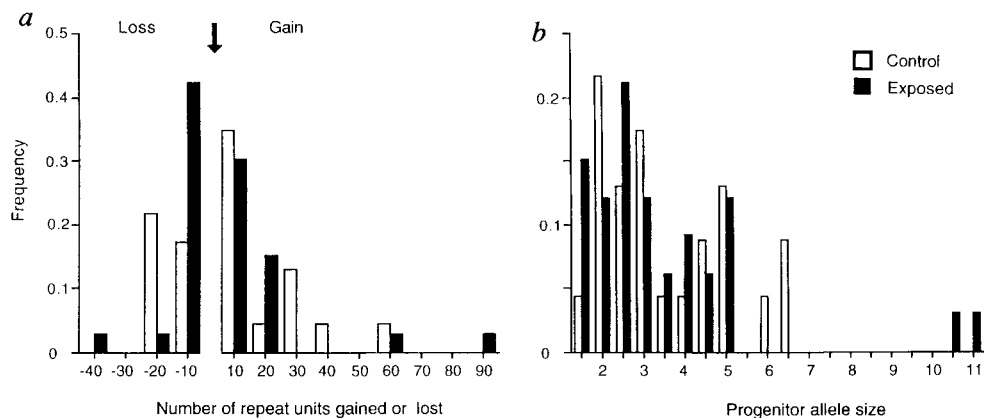


FIG. 4 ¹³⁷Cs surface contamination and mutation rate. a, Distribution of ¹³⁷Cs surface contamination in the place of residence of each Mogilev family analysed (data taken from ref. 30). b, Frequency of minisatellite mutation ($\pm$ s.e.m., probes 33.15, MS32 and CEB1) in offspring grouped according to surface contamination (0, control population).

FIG. 5 Characteristics of minisatellite mutants detected by four single-locus probes. *a*, Distribution of fragment size changes (Kolmogorov–Smirnov test, $P > 0.05$). *b*, Distribution of progenitor allele sizes for mutants (Kolmogorov–Smirnov test, $P > 0.05$). The progenitor allele was assumed to be the parental allele closer in size to the mutant allele⁹; progenitors were verified for MS31 and MS32 mutants by MVR–PCR^{17,18}.



direct targets of irradiation¹². The human haploid genome contains about 3×10^9 base pairs, and the average minisatellite scored by probe 33.15 has about 5,000 base pairs. The increase in mutation rate apparently caused by radiation was found to be about 0.01 per offspring band (probe 33.15; Table 1). If minisatellite mutations are initiated by double-strand breaks (DSBs)¹⁸, then this increase would require 6,000 extra DSBs per haploid genome, assuming that minisatellite loci are random targets; no more than 70 DSBs are induced per cell per 1 Gy of irradiation¹⁹. This discrepancy is even greater for CEB1, which shows an increase in paternal mutation rate in the Mogilev group of 0.13 per sperm. It therefore seems that the increase in mutation rate is not caused by targeted events (DNA damage induced directly at minisatellites), but rather results from non-targeted effects caused by radiation elsewhere in the genome. One possibility is that radiation induces some system in the germline that in turn stimulates minisatellite instability, for example by interacting with a rate-limiting step in the complex gene conversion-like pathway known to be involved in mutation at human minisatellites¹⁸. It remains to be seen whether this system alters the mutation process itself, as seen for somatic mutations in two human protein-coding loci, which show a marked change in structural basis between spontaneous and radiation-induced mutation^{20,21}.

Discussion

We have used five minisatellite probes to estimate the mutation rate after the Chernobyl accident and found a doubling in mutation frequency among offspring of irradiated parents that may be a direct consequence of radiation exposure. Data collected in Hiroshima and Nagasaki during the past 40 years from the children of atomic bomb survivors, using eight different indicators, do not provide evidence of any statistically significant

differences between exposed and control families^{22,23}. We therefore believe that the present study provides the first experimental evidence that germline mutation rates in humans can be increased by ionizing radiation. These data have been obtained by a new approach using hypervariable loci with spontaneous mutation rates at least 1,000 times higher than in most protein-coding loci. Evidence for germline-mutation induction was obtained from a small sample (only 552 individuals from both the control and exposed groups), which is substantially lower than the sample size needed to detect the same increase in mutation rate by standard genetic techniques²⁴.

The dose–response curve for radiation-induced minisatellite mutation remains unknown. Estimates of radiation dose in rural populations of the Mogilev district suggest an average thyroid exposure by ¹³¹I of about 0.185 Gy per person². In contrast, the individual radiation dose for external and internal chronic exposure to ¹³⁷Cs was estimated to be less than 5 mSv per year², a value far below that predicted from mouse data^{11–13,25} and current estimates of the doubling dose for humans²³. It therefore seems either that the observed increase in minisatellite mutation rate, if resulting from radiation, was caused by the initial acute ¹³¹I exposure, or that doses of chronic irradiation by ¹³⁷Cs have been substantially underestimated. Alternatively, it is possible that low doses of chronic irradiation are more effective in mutation induction than higher doses of acute irradiation^{26,27}. Additional population surveys are needed to test whether ionizing radiation does induce minisatellite mutation, and to investigate the relative impact of acute and chronic radiation exposure on germline instability.

Note added in proof: A similar study of minisatellite mutation in a relatively small number of families of atomic-bomb survivors from Hiroshima and Nagasaki has failed to show evidence for mutation induction following acute exposure³¹. □

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A galactic chimney in the Perseus arm of the Milky Way

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GALAXIES are surrounded by large haloes of hot gas¹ which must be replenished as the gas cools. This has led to the concept² of galactic 'chimneys'—cavities in the interstellar medium, created by multiple supernova explosions, that can act as conduits for the efficient transport of hot gas from a galaxy's disk to its halo. Here we present a high-resolution map of atomic hydrogen in the Perseus arm of our Galaxy, which shows clear evidence for the existence of such a chimney. This chimney appears to have been formed by the energetic winds from a cluster of young massive stars, and may currently have reached the stage of blowing out into the halo.

The interstellar medium of our Galaxy is known to exist in several phases, including a hot ionized medium which occupies cavities in the cooler neutral gas. In some cases, large cavities, or superbubbles, seen in our own and other galaxies are related to shells of cool material³. Heiles^{4,5} has catalogued a large number of Galactic shells based on analysis of low-resolution atomic hydrogen (H I) surveys of the Galaxy^{6,7}. These shells are thought to be produced by the combined action of winds from massive stars and sequential series of supernova explosions in OB associations over timescales of several tens of millions of years. Shells with sufficient energy should break through the thin disk of the Galaxy, thereby creating a channel for energy and material flow from the disk to the halo. Heiles⁸ has discussed the effect of this phenomenon on the evolution of the structure of the interstellar medium and how it could serve to energize the halo. Theoretical treatment of these so-called chimneys has been carried out by several authors^{9,10} but no clear observational example of a Galactic chimney with evidence of outflow had yet been obtained, primarily due to the low angular resolution of large-scale Galactic H I images.

As part of a pilot study for a large-scale, high-resolution survey of the H I emission from the Galaxy, we produced images of the Cassiopeia OB6 association region in the Perseus arm using the Synthesis Telescope (ST) at the Dominion Radio Astrophysical Observatory (DRAO). Figure 1 shows the resulting spectral line and continuum emission at 21 cm. Superimposed on the H I image are contours of ¹²CO (*J*₁ → 0) line emission as obtained by Digel *et al.*¹¹. Figure 1, top panel, shows an obvious cone-shaped cavity in the H I opening upwards, in a direction perpendicular to the Galactic plane, towards the region of lower density, as one would expect for a chimney. It sits directly above the W4 H II region, and the shape of its base matches the upper portion of that region quite well. W4 is ionized by the open star cluster OC1352 which contains nine O-type stars. Their location at the apex of the H I void (indicated by the red crosses) strongly suggests that they are responsible for creating the chimney. The chimney is taken to be at the same distance as W4 and OC1352 and a value of 2.2 kpc is adopted for the remainder of the discussion. Also present in the H I image is a filamentary arc within the chimney, near the upper latitude limit. This may be a remnant of the original wind-blown shell.

The V-shaped filament of H I emission seen near the centre of the chimney is suggestive of outflow towards higher Galactic latitudes. Two prominent CO clouds exist within the chimney; both are elongated in a direction away from the star cluster. The northern cloud lies at the base of the H I V-shaped 'streamers'. The streamers may represent previously molecular material that

TABLE 1 Properties of O stars in OC1352

Star	Spectral type	log $\dot{M}$ (M in $M_{\odot}$)	v_{∞} (km s^{-1})
BD + 60 504	O4I	-5.33*	2,600‡
BD + 60 502	O5III	-5.61*	3,350§
BD + 60 507	O5V	-5.77*	2,900§
BD + 60 497	O6V	-6.19†	3,600
BD + 60 513	O7V	-6.56†	2,900
IC 1805 113	O9V	-7.17	2,100
BD + 60 498	O9.5V	-7.36	2,000
BD + 60 499	O9.5V	-7.36	2,000
BD + 60 501	O6.5V	-6.72†	2,600

Where no reference is given for a mass-loss parameter that value has been evaluated theoretically based on the spectral type using empirical relations¹⁶.

* Ref. 14, † ref. 15, ‡ ref. 22, § ref. 21, || ref. 20.

has been dissociated and stripped from the molecular cloud by the outflow within the chimney. A velocity gradient of 11.5 km s^{-1} , starting at -36.8 km s^{-1} with respect to the local standard of rest (as are all other velocities in this Letter), exists along these streamers, with the material closer to the tips being more blue-shifted. A similarly oriented gradient of 6 km s^{-1} is present in the CO cloud. The velocity range of the CO cloud (-35.3 to -41.2 km s^{-1}) overlaps that of the H I streamers, indicating a progression from molecular to atomic gas as velocity becomes more negative. This could be caused by the material being accelerated as it moves away from the molecular cloud, in a direction inclined towards the observer. At 2.2 kpc, the mass of H I in the streamers is 430 solar masses. We do not know the inclination angle. But if we take 11.5 km s^{-1} as a lower limit for the relative velocity between the accelerated gas and the original molecular cloud material, the kinetic energy imparted to that material would be $> 6 \times 10^{47} \text{ erg}$ and the kinematic age, $< 4.5 \text{ Myr}$. The CO cloud is barely resolved in the direction perpendicular to the flow. Using the angular resolution of the data set (8.7 arcmin) as a lower limit for its size, it subtends a solid angle of $\sim 6.2 \times 10^{-2} \text{ sr}$ as viewed from the centre of the cluster of O stars. If the energy source accelerating the streamers is isotropic outflow from the cluster, the required energy outflow rate is $> 9 \times 10^{35} \text{ erg s}^{-1}$, a lower limit as only energy imparted to the atomic gas is taken into account.

Figure 2 is a schematic representation of the chimney, showing its relationship to the large-scale geometry of this region of the Perseus arm. The upper part of the figure shows the tilt towards the observer suggested by the blue-shifted flows of the CO cloud and the H I streamers, and, as the OC1352 stars are not heavily obscured, the chimney has been placed on the near side of the arm. The slight tilt of the cross-section of the Perseus arm represents the warp of the Galactic plane in that direction. The lower part of the figure shows the details of the chimney, including the nine O stars, the elongated molecular clouds, and the H I streamers and filamentary arc. The eastern wall of the H I void is traced by an ionized filament, seen faintly on the 21-cm continuum image (Fig. 1, lower panel), extending upwards from the north-eastern edge of W4. This filament probably represents chimney wall material that has been ionized by the stellar radiation field within the cavity. The ionized gas of W4 itself is shown outlining a cavity below OC1352.

The origin of supershells and theoretical considerations of the phenomena of blow-out and chimneys are generally discussed in terms of sequential supernova events, the effects of which are approximated by a continuous 'superwind'. However, over its lifetime, a massive star can release energy equivalent to a supernova explosion via a stellar wind. The spectral types, mass-loss rates ($\dot{M}$) and terminal wind velocities (v_{∞}) for the nine O-type

stars in OC1352 are listed in Table 1. These stars have a combined mechanical wind luminosity of $3 \times 10^{37} \text{ erg s}^{-1}$. This is sufficient to impart enough energy to the H I associated with the CO cloud for the production of the streamers. But can the O star winds alone also account for the chimney itself? The growth of a wind-driven shell in a uniform-density medium can be described by the simple analytical expression¹²,

$$R_s = 66 L_{38}^{1/5} n_0^{-1/5} t_6^{3/5} \quad (1)$$

where R_s is the shell radius in pc, L_{38} is the mechanical luminosity of the wind in units of $10^{38} \text{ erg s}^{-1}$, n_0 is the ambient density of the interstellar medium in cm^{-3} and t_6 is the age of the shell in units of 10^6 years.

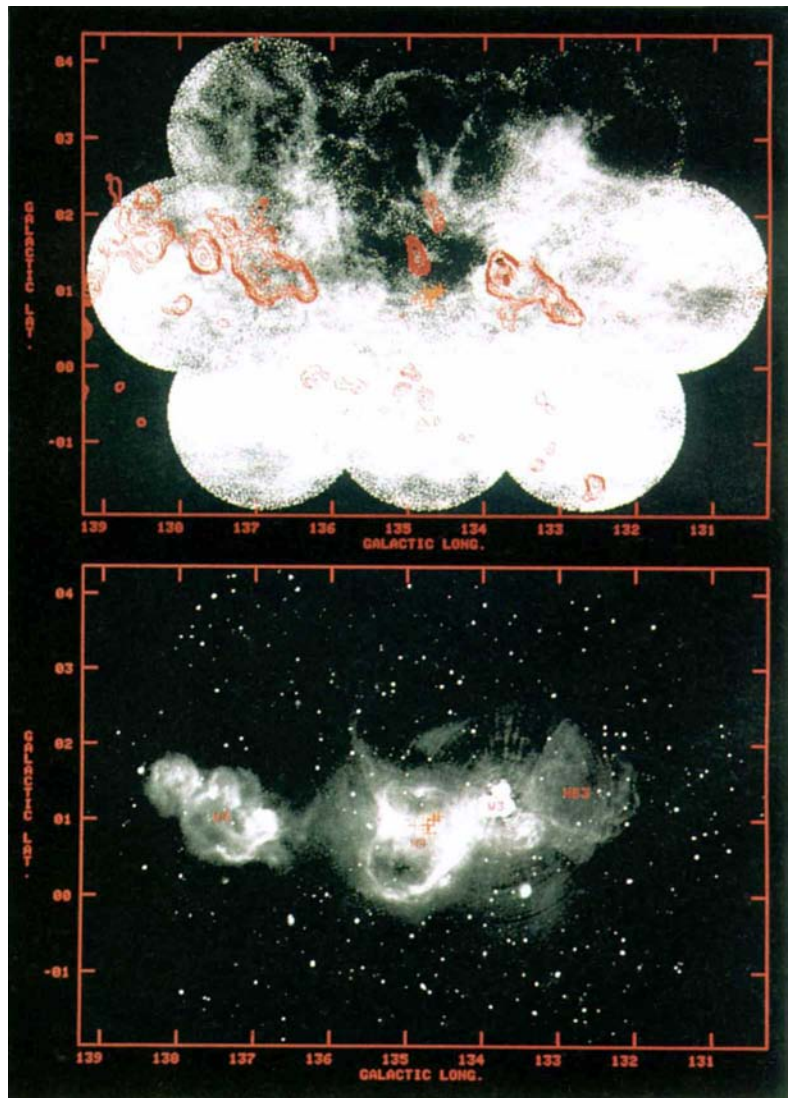
The cavity in the atomic gas corresponding to the chimney occupies a velocity interval of 14.8 km s^{-1} . To obtain an estimate of the density of ambient interstellar medium gas, we measured the average column density of H I over the same interval inside a 25.5-arcmin-wide latitudinal strip adjacent to the chimney. South of OC1352 there is a region of high column density, up to $26 \times 10^{20} \text{ cm}^{-2}$. Towards higher latitudes, the column density generally decreases, reaching $14 \times 10^{20} \text{ cm}^{-2}$ at Galactic latitude $b = +3.8^\circ$ after which there is an apparent sharp drop. The width of the chimney increases with latitude, reaching $\sim 120 \text{ pc}$ at $+3.8^\circ$, which is 110 pc above the star cluster. Assuming that the chimney has a depth equal to its width and that the adjacent gas extends over the same distance interval, then the H I density at the latitude

of the star cluster is 5 cm^{-3} . The density at the top of the chimney is $\sim 30\%$ lower. If we approximate the latitude distribution of H I as a uniform slab, using an average density of 4.4 cm^{-3} up to a height of 110 pc , with a low-density region above, we can invert equation (1) to derive the time required for a shell driven by the O star winds to blow out. For $L_{38} = 0.3$, the time required is 5.7 Myr . Note that this allows time for the creation of the H I streamers within the formation time of the chimney.

Of the nine O stars in OC1352 only BD + 60 504 (O4I) and BD + 60 502 (O5III) have left the main sequence. The other O5 star, BD + 60 507, has not. Using the expression for the main-sequence lifetime of stars with masses in the range of 30–80 solar masses given by Chiosi *et al.*¹³ and mass estimates appropriate to BD + 60 507 (refs 14–16), its main-sequence lifetime should be in the range of 3.7–4.3 Myr. Cluster age estimates by other authors^{17–19} also yield values of a few Myr. Thus the age of the stars is approximately consistent with the time required for their wind energy to create a chimney. The rough coincidence of the age of the cluster and the time required to blow out the chimney, as well as the presence of the H I filamentary arc above 110 pc , suggest that the blow-out of the chimney may be a very recent phenomenon.

The discovery of the Perseus chimney offers the opportunity to study in detail the structure and kinematics of a Galactic chimney and to investigate the circumstances of its formation. Although we have sketched out a plausible scheme for the creation of the

FIG. 1 The Cas OB6 association in the Perseus arm. Top panel, atomic hydrogen emission within a velocity interval of 1.6 km s^{-1} centred on -41.8 km s^{-1} with respect to the local standard of rest. Brightness temperatures $< 4.4 \text{ K}$ are in black, and those $> 31.6 \text{ K}$ are in white, with a linear scale in between. Contours of ^{12}CO brightness temperature¹¹, which has been integrated over a velocity interval of 6.5 km s^{-1} centred on -39 km s^{-1} with respect to the local standard of rest, are superimposed and plotted (red) at 0.2, 0.4, ..., 1.0, 1.5, ..., 3.0, 4.0 and 5.0 K. Bottom panel, radio continuum emission at 21 cm. The intensity scale varies linearly between 0 and 9 K. The image has been 'burned out' at high intensities to highlight some of the lower-intensity features; the actual maximum is 2.028 K at the peak of W3 (Galactic longitude $l = 133.7^\circ$, latitude $b = 1.2^\circ$). Southeast of W3 is a large filamentary loop of ionized gas, the W4 H II region. Further east is the W5 H II region (centred on $l = 136.9^\circ$, $b = 1.0^\circ$). Adjacent to W3 on the western side is the faint shell of the supernova remnant HB3 (centred on $l = 132.6^\circ$, $b = 1.5^\circ$). Ten overlapping fields were observed with the DRAO ST to produce the H I and radio continuum images, yielding a data set which combines a large field of view ($8^\circ \times 6^\circ$) with good spatial resolution ($\sim 1 \text{ arcmin}$). The large U-shaped cavity in the H I at top centre is the chimney blown by the young, massive stars in the open cluster OC1352 (shown by the red crosses). The upper portion of the ionized gas in W4 coincides with the bottom chimney. The V-shaped H I emission structure within the chimney is indicative of gas outflow up the chimney. Two prominent CO clouds are present within the cavity. Both are elongated in a direction pointing away from OC1352, suggesting an interaction with the energetic winds from the cluster stars. The northernmost cloud lies at the apex of the H I streamers. The other has a dense 'head' with a 'tail' stretching away from the centre of the cluster. The continuum image shows a bright knot of ionized gas just below the head, indicating that there is an ionization front on the side facing the stars.



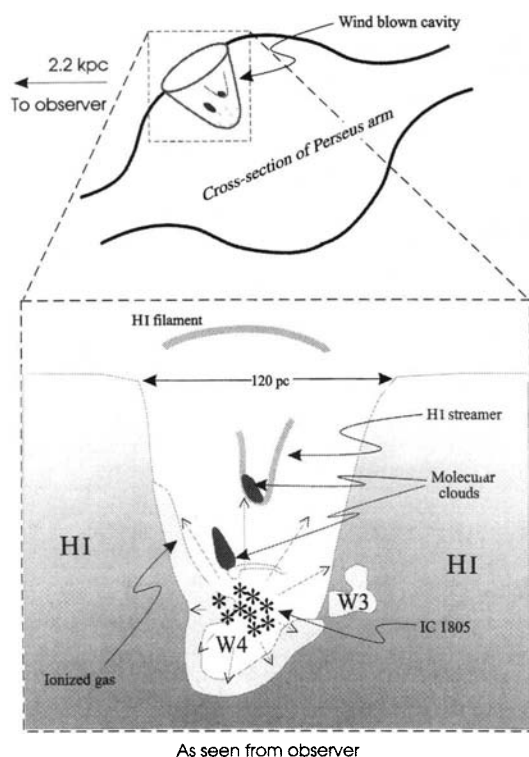


FIG. 2 A schematic representation of the chimney. The upper portion shows the suggested position of the chimney within the Perseus spiral arm. The observations indicate that the chimney is tilted towards the observer and on the near side of the arm. The lower portion shows the details of the chimney and the interactions between the various components within it. The chimney is assumed to be at the same distance as the W4 H II region based on the interaction of W3 and W4 (refs 23–25) and the coincidence in velocity space of the chimney and the disappearance of absorption features associated with W3. Distance estimates^{26,27} for these H II regions and the cluster OC1352 (indicated by the * symbols) in W4 range from 1.6 to 2.7 kpc; we adopt 2.2 kpc.

chimney, many questions remain unanswered. Hydrodynamic modelling should provide more accurate age estimates and energetics. Observations should be carried out to detect directly the hot gas flowing up the chimney. One way of doing this would be to search for evidence of hot, shocked gas at the interaction fronts of the flow with the molecular clouds. Within a few Myr, the massive stars in OC1352 will explode as supernovae, producing an expanding supershell. The efficiency of mass and energy transfer to the halo from these clustered supernovae will be enhanced by the pre-existence of the wind-blown chimney. If chimneys such as this are common features of clusters of massive stars, they may have a significant effect on the overall rate of energy and mass transfer from the disk to the halo. □

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The orbital evolution of the asteroid Eros and implications for collision with the Earth

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THE population of asteroids that cross the Earth's orbit is responsible for most of the terrestrial impacts of kilometre-size objects, of which there may be several per million years¹. About 150 Earth-crossing asteroids are known, although many more are thought to exist². Asteroids that come close to the Earth's orbit, but do not currently cross it, may also pose a threat if they evolve onto Earth-crossing orbits. The asteroid 433 Eros, with a diameter of ~ 22 km and a perihelion of 1.13 AU (where 1 AU is the average distance of the Earth from the Sun), is the second-largest near-Earth asteroid³. Here we report a study of the dynamical evolution of Eros's orbit over a period of two million years. We identify an orbital resonance with Mars that has the potential to perturb Mars-crossing asteroids, such as Eros, onto Earth-crossing orbits; of eight trial orbits that closely match Eros's present orbital parameters, three become Earth-crossing on the timescale of our simulations, and one of these hits the Earth after 1.14 Myr. Although our simulations indicate no significant danger of a catastrophic impact by this large near-Earth asteroid during the next $\sim 10^5$ years, such a collision is likely in the far future.

433 Eros does not currently cross the Earth's orbit, but it undergoes relatively frequent close encounters with Mars and long-range perturbations by the outer planets. The corresponding orbital changes may cause its perihelion to reach 1 AU within ~ 1 Myr (ref. 4). A collision with the Earth would have dire consequences. In the past 15 years, it has been realized that impacts by asteroids or comets exceeding a few kilometres in size have been major agents in the evolution of the biosphere, and cause a significant hazard to the future of the human civilization^{5,6}. Eros is about twice as big (that is, one order of magnitude more massive) as the extraterrestrial projectile that formed the Chicxulub crater about 65 Myr ago and probably caused the catastrophic K/T boundary extinction of living species^{7,8}. So investigating the future evolution of Eros's orbit, and estimating the chance that it will actually become an Earth-crosser and hit our planet, may give important clues to the occurrence of global impact catastrophes in the history of Earth.

How can we study the future (and past) evolution of Eros's orbit? The orbits of planet-crossing objects, including both near-Earth asteroids and comets, are very chaotic (with Lyapunov divergence times $t_L \approx 10^2$ yr), owing to the sensitive dependence

of the orbital changes caused by close planetary encounters on the pre-encounter orbital elements^{9,10}. A consequence of this is that the evolution of such objects cannot be studied by analytical tools, as orbital crossings and encounters would introduce singularities in the series developments which are used to represent the solutions of the equations of motion. Thus we decided to study the long-term dynamical evolution of 433 Eros by purely numerical integrations of the N -body gravitational problem.

These integrations were carried out with a Bulirsch–Stoer variable step-size technique¹¹, optimized for dealing accurately with close encounters, and also adapted by us for recording in an accurate way the miss distances corresponding to the encounters, despite the discrete time steps used by the program to compute the orbit and give the corresponding position and velocity vectors¹². As usual in studies of this type, we adopted a purely gravitational model of the Solar System, including all the planets except Pluto, the mass of Mercury being added to that of the Sun. The integration time always spanned 2 Myr in the future.

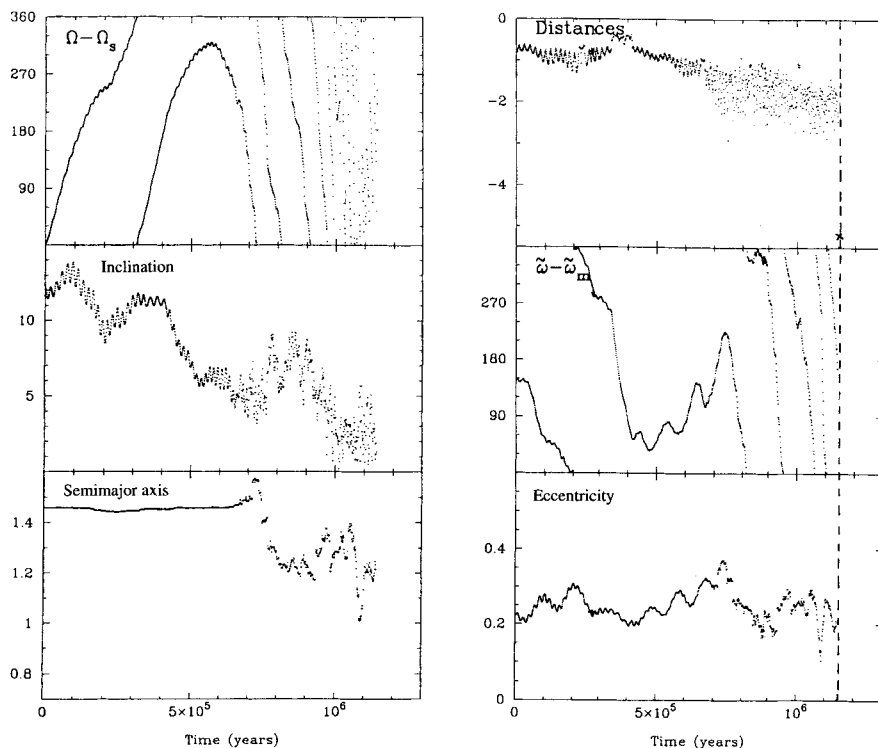
The strongly chaotic nature of the orbits implies that the results of numerical integrations cannot be seen as deterministic predictions of the evolution of the real objects, at least over times much longer than t_L . Indeed, planet-crossing orbits are very sensitive not only to the chosen initial conditions, but also to the assumed physical model of the Solar System, to the integration algorithm and to the round-off features of the computer used to run the integrations. Introducing small changes to any of these options implies that after a time span of several times t_L , the computed orbits completely 'forget' their common origins. They are just 'possible evolutions', useful in identifying the most important dynamical mechanisms which may affect the real orbit, or to make statistical estimates on the object's lifetime, encounter rate and so on. Thus in the case of Eros we studied not only the nominal orbit corresponding to the initial conditions listed in the ephemerides, but also a sample of 'clone' orbits. Three clones (called Eros Ia, Ie and Ii) were obtained by a relative variation of 10^{-3} (one at a time) of the starting semimajor axis, eccentricity and inclination of the nominal orbit (Eros I); four more clones (Eros II, IIa, IIe, Ili) were then obtained by performing the same integrations again but on a different computer. Thus we obtained eight orbital evolutions overall.

Over an integration time of a few times 10^5 yr, the results of our integrations confirm the previous finding⁴ that although Eros's orbit keeps Mars-crossing and undergoes only shallow Earth approaches (with closest approach distances >0.03 AU), the orbital elements do not show strong changes related to planetary encounters. But on a longer time span, we find two different classes of dynamical behaviour: three clones (Eros I, Iie and Ili) become Earth-crossers within 2 Myr, and one of them (Eros I) is even observed to collide with the Earth during the integration time. The other five clones remain always just Mars-crossers, and their future orbit is not affected by encounters with the Earth. Figure 1 shows in detail the behaviour of the Earth-colliding body, whereas the evolution of all the three Earth-crossing clones in the semimajor axis versus eccentricity plane is represented in Fig. 2.

The three bodies becoming Earth-crossers show fairly similar patterns. However, two of them (Eros Iie and Ili) reach a perihelion distance $q = 1$ AU after more than 1.5 Myr, whereas Eros I gets into the Earth-crossing region after only 0.55 Myr and eventually hits the Earth at $t = 1.14$ Myr. During their Mars-crossing phase, in several instances these bodies get temporarily locked over $\sim 10^5$ yr in the 4:7 mean motion resonance with the Earth (with the asteroid completing 4 revolutions around the Sun, while the Earth makes 7 of them). This resonance results in a 'protection mechanism', which prevents close Earth encounters and slows down the dynamical evolution in the Mars-crossing region; such protection mechanisms are quite common for near-Earth asteroids, though they are only temporary⁴. When the first close encounter with the Earth occurs, the orbits are located in a region of the orbital element space where two secular resonances¹³ (implying very close orbital precession rates for the asteroid and a planet) overlap each other, one involving Saturn, the other Mars (see Fig. 1). The resonant perturbations result in a decrease of the inclination and an increase of the eccentricity, leading the orbits to enter and stay in the Earth-crossing region ($q < 1$ AU). Such a significant effect on the orbits of near-Earth asteroids by a secular resonance with a terrestrial planet (here Mars) has not, to our knowledge, been pointed out before, and could provide an important dynamical mechanism by which asteroids in the Mars-crossing region eventually achieve Earth-crossing orbits.

After having reached the $q < 1$ AU region, Eros I undergoes a

FIG. 1 Evolution of the orbital elements of Eros I until it collides with the Earth. Besides semimajor axis (in AU), eccentricity and inclination (in degrees) versus time, the figure also shows (in degrees) the two critical arguments $\tilde{\omega} - \tilde{\omega}_M$ and $\tilde{\Omega} - \tilde{\Omega}_S$ of the v_4 and v_{16} secular resonances ($\tilde{\omega}_M$ being the perihelion longitude of Mars and $\tilde{\Omega}_S$ the nodal longitude of Saturn), and the closest approach distances to the Earth (in AU, logarithmic scale) recorded during a sequence of successive 10^3 yr intervals (the cross and the vertical dashed lines in the right-hand plots mark the collision event). The effect of v_4 (with the asteroid's apsidal rate $\dot{\omega}_M$ equal to that of Mars, $\dot{\omega}_M$) is to increase secularly the eccentricity and thus to bring the orbit closer to that of the Earth, whereas v_{16} (with the asteroid's nodal rate $\dot{\Omega}$ equal to that of Saturn, $\dot{\Omega}_S$) leads to a decrease of the inclination, therefore increasing the probability of effective encounters with the planets.



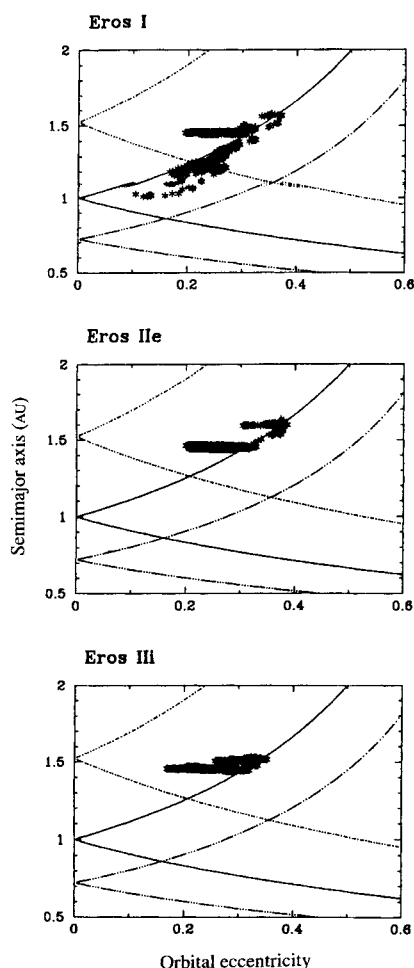


FIG. 2 The evolution of the three Eros Earth-crossers in the semimajor axis (a , in AU) versus eccentricity (e) plane. (See text for I, IIe, III nomenclature.) The dot-dashed, full and dotted lines correspond to perihelion and aphelion distances equal to the semimajor axes of Venus, the Earth and Mars, respectively. All the three bodies get inside the full lines delimiting the Earth-crossing region, although only Eros I spends most of the time there.

number of close Earth encounters, down to a few times 10^{-3} AU, and as a result the semimajor axis shows an irregular random walk. Eventually, a collision (minimum distance 6×10^{-6} AU from the Earth's centre) occurs 1.14 Myr after the beginning of the integration.

After detecting this collision, we tried to use our integrations to derive some statistics about the occurrence of close Earth encounters by the Eros clones, which may be extrapolated to estimate Eros's collisional lifetime. This is possible because over times much longer than t_L , the outputs of the eight integrations are independent of each other. Considering only the clone (Eros I) which spends most of the time in the Earth-crossing region, the frequency of encounters closer than ~ 0.01 AU is roughly proportional to the square of the closest-approach distance, and extrapolating this trend down to the Earth's radius we obtain a lifetime of ~ 120 Myr, fairly typical for an Apollo-like orbit¹. But considering that seven of the eight Eros clones spend no (or just a small fraction of) time in the Earth-crossing region, an average over all the eight integrations leads to a much longer lifetime, $\sim 1.4 \times 10^9$ yr. Thus, although we cannot exclude the chance that Eros will strike the Earth within the next few Myr (as in our Eros I integration), statistically we expect a much longer lifetime, with most of the orbital evolution occurring in the Mars-crossing region.

Seven of the eight clones spent the whole integration time with their aphelion in the main asteroid belt, beyond ~ 1.7 AU. The only clone for which this did not happen is Eros I, which underwent an early Earth impact. Our results do not provide information over a time span comparable to Eros's lifetime, which is 10^8 – 10^9 yr with respect to both an Earth impact and a disrupting collision by another asteroid¹. However, the real Eros is unlikely to have spent most of its lifetime with the aphelion fully decoupled from the asteroid main belt, so when the cratering rate on the surface is inferred from images to be obtained by the NEAR space mission in 1999 (refs. 14, 15), a main-belt projectile population will have to be assumed to estimate its age.

From our integrations we conclude that 433 Eros has a significant probability (of the order of 50%) to become an Earth-crosser and even collide with our planet on a timescale of 10^8 – 10^9 yr. This shows that impacts with asteroids much bigger than the K/T boundary impactor are not unlikely to have occurred (or to occur in the future) during the history of our planet, although there is no chance of such an event over the next 10^5 yr. Our integrations also show that such large impactors may be delivered through a peculiar dynamical mechanism, involving several resonances (including a secular one with Mars), which probably affect the dynamical evolution of many other near-Earth asteroids. $\square$

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Manipulation of an atomic beam by a computer-generated hologram

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TECHNIQUES for manipulating neutral atoms are valuable tools for the investigation of surfaces, and hold promise for the fabrication of atomic structures for technological applications. The ability to position individual atoms with the accuracy of a crystal lattice constant has been demonstrated¹ using the scanning tunnelling microscope (STM). On the other hand, manipulation with lasers offers a means of controlling atoms in bulk, although it lacks the positional accuracy of the STM. The ability to generate ultra-cold atoms^{2–5} using lasers has opened new possibilities: because of their long de Broglie wavelengths, cold atoms are amenable to interferometric manipulation, such as deflection by a grating^{6,7} and focusing by a Fresnel lens⁸. Here we demonstrate a poten-

tially more flexible approach to atomic manipulation based on holographic principles. We pass a beam of ultra-cold metastable neon atoms through a computer-generated hologram that encodes the Fourier transform of a desired atomic pattern. Diffraction of the atomic beam by the hologram then reconstructs the pattern, in a manner analogous to optical holography. This approach should in principle enable the imposition of arbitrary intensity and phase information onto an atomic beam.

Holography is a technique for reproducing an optical wavefront by passing an optical beam through a phase and/or contrast object called a hologram. The hologram can be fabricated through photographic recording of two interfering optical beams, or by numerical calculation of the interference pattern by computer. To reduce the amount of calculation, computer holography often uses approximation. One such approximation is the binary hologram, in which the hologram takes a binary value, either 100% transparent or 100% opaque. This hologram can be directly translated to a hologram for atomic de Broglie waves, by cutting out the pattern on a film that is equal to the pattern of the 100% transmission area of the optical binary hologram. A monochro-

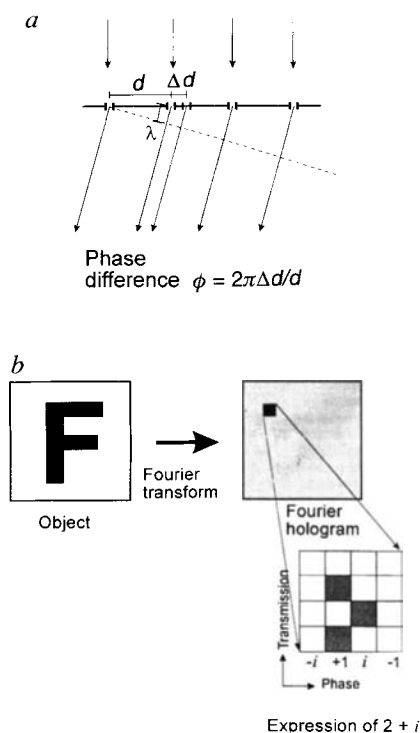


FIG. 1 Diagrams showing the principles of our technique. The hologram is designed to generate the Fourier-transformed amplitude of the object on a reference plane that is tilted from the hologram surface by an angle of λ/d along the x-axis. The reference plane coincides with the wavefront of the first-order diffracted wave from a regular array of slits having the interval d . The relative phase between the incident and diffracted waves changes in units of 2π at successive slit positions. Therefore the complex field amplitude $E = |A| \exp(i\phi)$ at some point (cell) on the reference plane can be generated by shifting the position of the slit by $\Delta d = \phi d / (2\pi)$ from the regular position and adjusting its length proportional to $|A|$ (panel a). In an actual hologram, a cell is divided into 4×4 subcells, and the four positions along the x-axis express the phase of $\phi = 0, \pi/2, \pi$ and $3\pi/2$, respectively. To express the imaginary part of the amplitude, $E = a + bi$, holes proportional to $|b|$ are open at the $\phi = \pi/2$ or $\phi = 3\pi/2$ position depending on the sign of bi . To express the real part, holes are open on the $\phi = 0$ or $\phi = \pi$ position. Because there are 4 subcells on each phase position, both a and b can take an integer value between -4 and 4 . Panel b shows an example of the amplitude $E = 2 + i$. We normalized the amplitude of the Fourier transform so as to minimize the number of unsupported closed subcells by keeping the relative number of the open subcells at $\sim 10\%$. The percentage of unsupported subcells was thus $< 0.01\%$.

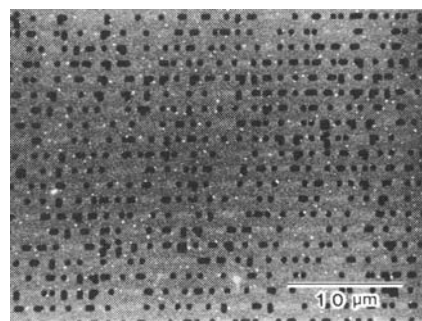


FIG. 2 Scanning electron microscope image of the hologram surface. The area of the SiN membrane is 1.5×1.5 mm, its thickness is 100 nm. Through-holes were created by one minute of CF_4 dry etching with an incident r.f. power of 50 W and a bias voltage of 200 V. The size of the subcell was $0.3 \mu\text{m}$. The area of a complete hologram was therefore $153.6 \times 153.6 \mu\text{m}$. Black squares indicate through-holes.

matic atomic wave reconstructs an atomic pattern that is the same shape as the optical hologram.

The hologram we used in this experiment was a Fourier hologram, which produced the Fourier-transformed wavefront of the object. When the hologram is illuminated with a plane wave, the far-field pattern of the diffracted wave produces an image of the object. If a focusing lens is placed after the hologram, the image is formed on the focal plane. We used a digitizing technique similar to that developed by Lohman *et al.*⁹ The principles of the method are shown in Fig. 1. The object that we used was a transparent F-shaped pattern, in which the transparent portion had a constant amplitude and random phase distribution. The object was represented by the complex transmission amplitude at points on the 128×128 matrix covering the F. The two-dimensional array of numbers was Fourier-transformed using a fast Fourier transform (FFT) algorithm, and the resulting 128×128 complex numbers were used as the transmission function of the 128×128 areas (cells) of the Fourier hologram. The transmission function of each cell of the hologram was expressed by a matrix of 4×4 subcells¹⁰. (Details of this method are given in Fig. 1 legend.)

A 100-nm-thick silicon nitride (SiN) membrane was used for the hologram. The binary pattern was transferred to a resist (ZEP, Nippon Zeon Co.) on the SiN membrane with an electron-beam writing system. Subsequent CF_4 plasma etching created through-holes in the membrane. A scanning electron micrograph of the hologram is shown in Fig. 2. The size of the subcell $d/4$ was $0.3 \times 0.3 \mu\text{m}$ square, so the size of the entire hologram was $153.6 \times 153.6 \mu\text{m}$. To increase the intensity of the deflected beam, the same pattern was repeated 10 times along the x and y directions, making the overall size of the hologram 1.5×1.5 mm.

A schematic diagram of our experiment is shown in Fig. 3. The ultra-cold Ne atomic beam was generated by the method reported¹¹ by one of the authors. The $1s_5(3s^3P_2)$ metastable ^{20}Ne atoms generated by the d.c. discharge were deflected, and then slowed in the Zeeman slower by a counter-propagating laser beam (of 640 nm wavelength) which was in resonance with the closed transition $1s_5-2p_9$. A spatially varying axial magnetic field was applied to compensate the Doppler shift of the atoms. At the end of the slowing stage was a magneto-optical trap with a four-laser-beam configuration, and the cooled $1s_5$ atoms were trapped around the point of zero magnetic field. The cloud of Ne atoms in the trap was ~ 0.3 mm in diameter, and the one-directional average velocity of the atoms was 20 cm s^{-1} . A focused 598-nm laser illuminated the Ne atomic cloud, inducing transition from the $1s_5$ to the $2p_5$ state. Half of the atoms in the $2p_5$ state decayed to the $1s_3(3s^3P_0)$ metastable state by spontaneous emission. The rest decayed to the ground state by cascade emission of two spontaneous photons. Both ground-state and $1s_3$ atoms were freed from the trapping force and dropped vertically, under the influence of

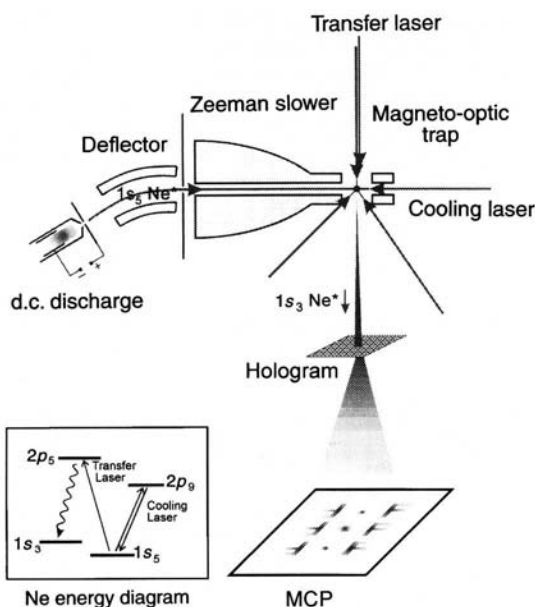


FIG. 3 The experimental apparatus. A beam of metastable Ne atoms in the $1s_5$ state is generated by a discharge. The beam is deflected 10^{-1} radians by using the recoil momentum of scattered laser photons in resonance with the cooling transition $1s_5-2p_9$. This collimates the beam and separates the ground-state Ne atoms. The beam passes through the Zeeman slower (see text); after the slowing stage a quadrupole magnetic field is formed, and the $1s_5$ metastable Ne atoms are trapped in the centre with the help of four resonant laser beams that are directed towards the centre. The temperature of the Ne atoms in the trap is typically $50\text{ }\mu\text{K}$. The second laser, emitting at 598 nm , is resonant to the transition $1s_5-2p_5$; absorption of a laser photon, followed by spontaneous emission, transforms the internal state of Ne atoms from $1s_5$ to $1s_3$ (see the energy-level diagram). The $1s_3$ atoms are free from the trapping forces and, pulled by gravity, drop nearly vertically. The hologram is placed 40 cm below the trap, and the $1s_3$ atoms diffracted by the hologram are detected 45 cm below the hologram by using a microchannel detector equipped with a fluorescent plate (MCP). The position of each atom detected by the MCP is recorded and accumulated by a computer.

gravity. Because the ground-state atoms were insensitive to the micro-channel-plate (MCP) detector, we detected only the $1s_3$ atoms. The hologram was placed 40 cm below the trap, and was mounted on top of a 0.2-mm diameter diaphragm. The size of the diaphragm limited the resolution of the image of the Fraunhofer hologram. The position of the hologram was not adjusted because any small portion of the hologram could produce the same image. The average atomic velocity at the grating was 2.8 ms^{-1} , corresponding to a de Broglie wavelength λ of 7.1 nm . The acceleration due to gravity reduced the relative velocity spread to $\sim 0.28\%$. To detect the Fraunhofer diffraction pattern from the hologram, a MCP detector was placed 45 cm below the hologram (see Fig. 3).

Figure 4 shows the reconstructed pattern. The data was accumulated for 10 h , and the total number of spots on the figure was $\sim 6 \times 10^4$. The diffracted beam consisted of atomic de Broglie waves with different diffraction orders (N, M) along the x and y directions, where the approximate diffracted angles of the (N, M) wave were $N\lambda/d$ and $M\lambda/d$ along the x and y directions, respectively. The waves with $N = 0$ did not carry phase information and gave spots equivalent to the diffraction from a grating with a pitch of d . Their spot size was the measure of the resolution of the reconstructed pattern. An additional weak spot just below the ($0, 0$) spot was produced by 74-nm vacuum ultraviolet photons generated at the trap when the $1s_5$ atoms decayed to the ground state. The first-order waves with $N = 1$ carried correct phase information and reproduced the pattern of the original object. The waves with $N = 4n + 1$ carried the same phase as the wave of $N = 1$, and therefore reproduced the same pattern. Their inten-

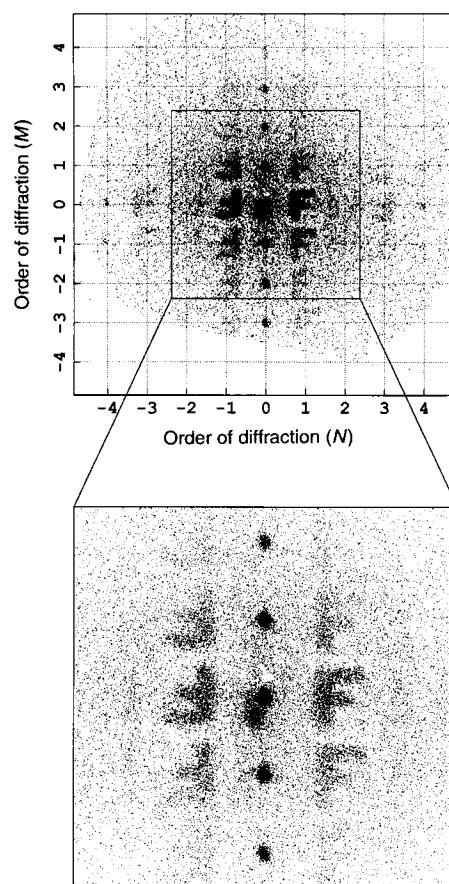


FIG. 4 The reconstructed image. The accumulated time is 10 h , and the figure contains about 6×10^4 atoms. The area of the atomic image is determined by the diffraction angle from a single subcell, and the separation of the images between two successive diffraction orders is determined by the size of the cell $d = 1.2\text{ }\mu\text{m}$. This separation is 2.18 mm . See text for discussion of the details of the image.

sity was less than that of ($1, 0$), because the phase variation inside the subcell partially cancelled the wave. The phase of the waves with $N = 4n - 1$ is conjugate to that of waves with $N = 1$. The reconstructed pattern therefore showed an inverted shape. The waves with $N = 4n + 2$ have a relative phase difference of π between two adjacent subcells, and should disappear when averaged over the entire hologram. The waves with $N = 4n$ or $M = 4n$ with $n \neq 0$ should also disappear, because the phase variation inside a subcell amounts to $2n\pi$. Weak spots at $(\pm 4, 0)$ and $(0, \pm 4)$ resulted either from the irregularity of the open subcell or from the change in the transmission characteristics of Ne atoms near the edge of the open subcell. The observed pattern of Fig. 4 reflected the above characteristics. As each cell was divided into 4×4 subcells, clear images were observed up to diffraction order ± 2 . The background of the image also covered the same area. The measured intensity of the ($0, 4$) wave relative to that of the ($0, 0$) wave was less than 1% . This shows that neither distortion of the hole nor the edge effect of the transmitting Ne atoms caused serious problems when imaging at the hole size of $0.3\text{ }\mu\text{m}$.

In the present experiment we did not use a focusing lens for imaging, and the resolution was limited by the diameter of the atomic beam. However, it is possible to combine the function of a focusing lens into the hologram. In such a hologram, the resolution is determined by the same rule as applies to an optical lens. The binary hologram does not control the phase and amplitude of the wave inside a hole. When the hologram is the sole component for atomic-beam manipulation, therefore, the practical limit is

approximately the minimum size of through-holes, which is in the range 10–100 nm. This means that the practical resolution of the holographic manipulation technique is of the same order as that of proximity manipulation. The size of the atomic source and the velocity spread of the atoms are not the real limiting factors of resolution, as the coherent portion of the atomic beam may be removed either by a diaphragm or by a velocity-selective chopper, though this reduces the intensity of the atomic beam. The number of atoms that contribute to the imaging is limited by the theoretical maximum diffraction efficiency (6.25%) of a two-dimensional amplitude hologram. This is a disadvantage for an imaging device. However, the holographic technique described here is an example of the more general technique of neutral atom manipulation. It can in principle construct an arbitrary wavefront of an atomic de Broglie wave retaining both intensity and phase information. When only the intensity of the image is required, it is possible to disperse the information of each object point over entire hologram surface, as we did in the present experiment. This makes the image very resistant to defects in the imaging component. Atomic holography is not limited to the generation of a fixed pattern with a fixed diffracting component. By combining it with other manipulation techniques, it should be possible to make a real-time moving pattern of atoms, or a device to investigate the three-dimensional phase and amplitude characteristics of an atomic de Broglie wave. □

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Photodegradation of methylmercury in lakes

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METHYLMERCURY can accumulate in fish to concentrations that threaten human health¹. Fish methylmercury concentrations are high in many reservoirs² and acidic lakes³, and also in many remote lakes^{4,5}—a fact that may be related to increased atmospheric deposition of anthropogenically mobilized mercury during the past few decades⁶. Although sources of methylmercury to lakes and reservoirs are known⁷, in-lake destruction has not been demonstrated to occur at the low concentrations found in most water bodies. Here we report *in situ* incubations of lake water that show that methylmercury is decomposed by photo-

degradation in surface waters. This process is abiotic and the rate is first-order with respect to methylmercury concentration and the intensity of solar radiation. In our study lake, the calculated annual rates of methylmercury photodegradation are almost double the estimated external inputs of methylmercury from rain, snow, streamflow and land runoff, implying the existence of a large source of methylmercury from bottom sediments. Photodegradation could also be an important process in the mercury cycle of other aquatic systems. This discovery fundamentally changes our understanding of aquatic mercury cycling, and challenges the long-accepted view that microbial demethylation dominates methylmercury degradation in natural fresh waters.

Recent development of highly sensitive analytical capabilities⁸ have enabled us to examine the stability of methylmercury in lake water at naturally occurring, low concentrations. At the Experimental Lakes Area (ELA) in northwestern Ontario, we incubated lake water in Teflon bottles (which transmit sunlight⁹) and found that methylmercury concentration decreased in the sunlight but not in the dark (Fig. 1a). Filtering the lake water with 0.45-µm filters, which removed all photosynthetic organisms and most bacteria, had no effect on the photodegradation rate (Fig. 1b). The fact that the reaction occurred in filtered water suggested that the photodegradation was abiotic. To confirm the abiotic nature of the reaction, we conducted an experiment using unfiltered, sterilized lake water and found that sterilization did not inhibit photodegradation (Fig. 1c).

The light-dependence of the reaction was demonstrated further in an experiment in which one set of bottles was exposed to sunlight, a second set was dark, and a third set was moved from dark to light during the experiment. Loss of methylmercury occurred only during the periods when bottles were exposed to sunlight (Fig. 1d).

We also determined that photodegradation rates were first-order with respect to methylmercury concentrations (Fig. 2a) and to levels of photosynthetically active radiation (PAR, Fig. 2b). The lake water used in all of these experiments (Figs 1 and 2) had a pH of 6.1–6.3 and a dissolved organic carbon (DOC) concentration of 1,190–1,470 µmol l⁻¹. Several comparisons of photodegradation rates in different ELA lakes spanning a range of water chemistry (for example DOC 250–1,210 µmol l⁻¹; pH 6.0–7.5; alkalinity 127–143 µeq l⁻¹) showed no differences in rates due to water chemistry (P.S., C.A.K., J.W.M.R. and A.R.M., unpublished data).

In situ incubations showed that methylmercury photodegradation rates decreased with depth below the lake surface, and corresponded closely to the exponential decrease in light intensity with depth (Fig. 3). Because we have shown that methylmercury photodegradation is consistent with standard rate laws for photochemical reactions, it should be possible to add this process to mechanistic models of mercury cycling in lakes and reservoirs¹⁰.

The end-products of methylmercury photodegradation in natural waters have not been identified. In laboratory experiments done at relatively high methylmercury concentrations (micrograms per litre), a variety of mercury and carbon species are photo-produced¹¹. In natural waters, however, the relatively low concentrations of methylmercury (0.05–3.0 ng l⁻¹ in ELA lakes and streams) are bound with complexing agents which are also present only in trace quantities¹². Therefore, the chemistry of the reaction in natural waters may be different than our laboratory experiments.

It is important to understand the difference between the process of photodegradation of methylmercury described here and the process of photoproduction of elemental mercury (Hg⁰; ref. 9). Photoproduction of Hg⁰ is thought to occur primarily by reduction of inorganic mercury (Hg²⁺; refs 13, 14), and flux of Hg⁰ to the atmosphere is an important mechanism by which mercury is lost from lakes¹⁴. Photodegradation of methylmercury may or may not produce Hg⁰. If it does, the estimated production rate of Hg⁰ in our study would be 1.5 µg m⁻² yr⁻¹. This is similar to the flux of Hg⁰ to the atmosphere measured in two previous studies of

lakes^{14,23}, but smaller than Hg^0 production rates in another study⁹. Of course, identification of methylmercury photodegradation end-products is necessary for a realistic interpretation of these rate comparisons. Even so, the immediate significance of methylmercury photodegradations that it decreases the concentration of

methylmercury, which is the form of mercury that is most easily accumulated in fish and that is the most toxic to fish consumers.

Two lines of evidence show that abiotic photodegradation is a much more important process in epilimnetic lake water than is biological demethylation, which can occur in the dark. First, our experiments repeatedly showed that methylmercury concentrations in unfiltered water were stable in the dark (for example, Fig. 1a), indicating that biological demethylation was either absent or was occurring at rates undetectable with our methods. Second, biological demethylation can be detected in lake water using ^{14}C -methylmercury at very high ($1,900 \text{ ng l}^{-1}$) concentrations (incubated in the dark), but the turnover rate measured is about 350 times slower than it is for photodegradation¹⁵. The data we present here therefore call into question the long-accepted view that biological demethylation is the dominant removal mechanism of methylmercury in lake water. However, biological demethylation may be important in sediments where methylmercury degradation rates measured using ^{14}C -methylmercury in the dark are much higher than in the water column^{15,16}.

Given that numerous studies of demethylation using ^{14}C -methylmercury have been done in the past¹⁷, one may question why photodegradation has not been observed before. The reason is that in all previous studies the samples were incubated in the dark¹⁷.

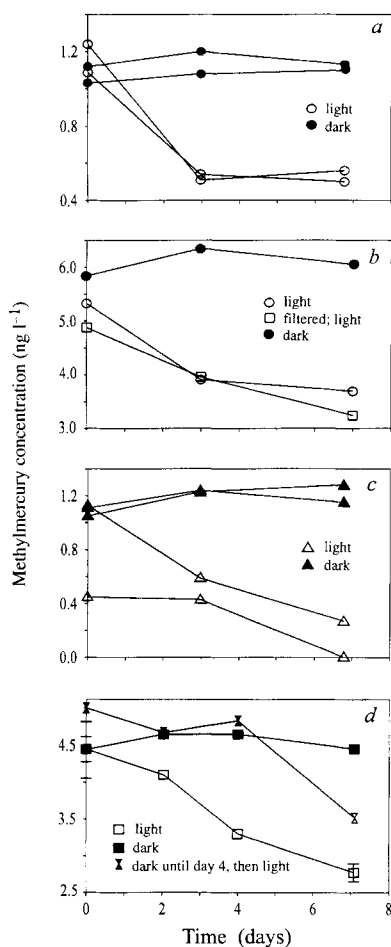


FIG. 1 Methylmercury concentrations in lake water incubated in light and dark (aluminum-foil covered) Teflon bottles on the surface of the lake. Each panel represents a separate experiment in which all bottles were incubated under the same light and temperature conditions. The experiments were conducted on different dates. Water treatments: a, unfiltered water; b, unfiltered water incubated in the light and the dark, and filtered water incubated in the light; c, unfiltered, sterilized water; d, filtered water in the light and the dark, or transferred from dark to light. In a and c, there were two sets of bottles incubated for each treatment. The lines represent separate sets, with each data point representing a single analysis from a single bottle incubated. The low initial value for one of the light treatments in c is due to analytical error. The data points in d represent duplicate analyses for a single bottle at each point in time, and range bars indicate where these duplicate analyses are larger than the data points.

METHODS. All sampling, manipulation and analyses of water were performed using ultra-clean techniques⁸. Water was incubated with a 50% headspace under the following temperature (mean daily) and light conditions: a and c, 17.5°C and $23 \text{ E m}^{-2} \text{ d}^{-1}$; b, 16.5°C and $21 \text{ E m}^{-2} \text{ d}^{-1}$; d, 20.0°C and $25 \text{ E m}^{-2} \text{ d}^{-1}$. For b and d, the concentration of methylmercury was elevated by addition of a methylmercuric chloride stock solution (made using distilled water). For c, water and bottles were sterilized by autoclaving, and were not opened until analyses. Lake water used for these experiments had a pH of 6.14, a dissolved organic carbon concentration of $1,410 \mu\text{mol l}^{-1}$ and a chloride concentration of 0.4 mg l^{-1} . Methylmercury concentration was determined by a distillation extraction, aqueous phase ethylation and atomic fluorescence detection⁸. A control experiment ensured that methylmercury did not absorb on the Teflon bottles: after incubation, acid ($9 \text{ N H}_2\text{SO}_4$) was added to sample bottles to a final concentration of 0.09 N . No methylmercury was released back into solution (data not shown).

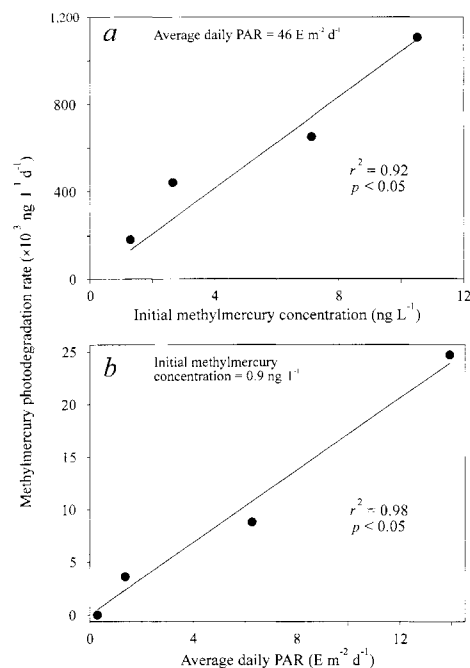


FIG. 2 a, The effect of initial methylmercury concentration on the photodegradation rate (the light level is the same for all incubations). r^2 , square of the correlation coefficient; p , level of significance. b, The effect of light intensity on methylmercury photodegradation rate. The initial methylmercury concentration is the same in all bottles.

METHODS. a, Unfiltered, methylmercury-amended, replicate lakewater samples were incubated on the lake surface for 4 days. For each of four methylmercury concentrations, there were three bottles and one bottle was analysed (in duplicate) on days 0, 2 and 4. Rates given were calculated by linear regression on the three time points. b, Bottles containing filtered lakewater were incubated at discrete depths to obtain different light levels. The PAR at each depth was calculated using surface radiation, and the average light extinction coefficient. This coefficient was determined with a LiCOR quantum sensor from nine light profiles taken during the incubation period. The surface bottles were incubated for 16 days and all others for 21 days because of lower light levels. The lake temperature ($^\circ\text{C}$) range at each incubation depth is as follows: 0 m, 9.2–15.2; 1 m, 9.3–15.3; 3 m, 9.3–15.4; 5 m 9.4–15.4; 9 m 9.5–14.1. Replicate bottles were incubated at each depth, and were collected and analysed (in duplicate) on three dates. Rates given were calculated from the average concentration on each date by linear regression of the three points in time.

To estimate whether the observed photodegradation rates could have a significant effect on the methylmercury concentration in lakes and reservoirs, we calculated a methylmercury turnover rate for the epilimnion of a typical ELA lake (Lake 240) during summer stratification of 1994. We used the following Lake 240 data: (1) the average epilimnetic methylmercury concentration (0.07 ng l^{-1}), (2) the average incident PAR ($35 \text{ E m}^{-2} \text{ d}^{-1}$; refs 18, 19) and (3) the average extinction coefficient for PAR (0.5 m^{-1}) in the epilimnion (0–4 m depth). We also used the relationships between photodegradation rate and methylmercury concentration (Fig. 2a), and between photodegradation rate and light intensity? (Fig. 3). From these data we estimated that the average photodegradation rate constant in Lake 240 was 0.043 d^{-1} for the ice-free stratified season. This means that the methylmercury in the epilimnion of Lake 240 would be replaced about seven times during the period of the summer stratification.

We made a preliminary comparison of the importance of photodegradation relative to the annual masses of methylmercury flowing into and out of Lake 240 at ELA (Fig. 4). Using previously published²⁰ and our unpublished data, we estimated that the total methylmercury input (direct deposition, stream flow, and runoff) was $\sim 370 \text{ mg yr}^{-1}$ and outflow was $\sim 140 \text{ mg yr}^{-1}$. Thus, if photodegradation is not included in the methylmercury cycle (Fig. 4a), one would conclude that there is net flux of methylmercury from the water column (net destruction and/or storage). But when photodegradation is included (Fig. 4b), there must be an in-lake source of methylmercury of $\sim 450 \text{ mg yr}^{-1}$. Because the entire water column of Lake 240 is oxic, and because mercury methylation is primarily an anoxic process²¹, this in-lake source is expected to be the sediments at a rate of $\sim 1 \mu\text{g m}^{-2} \text{ yr}^{-1}$. This mass-balance shows that during the year of our study, the flux from sediments must be similar in importance to external inputs as a source of methylmercury to this lake.

Knowledge of photodegradation may be useful in the design of methods to mitigate methylmercury problems. For example, where effluent water contains high concentrations of methylmercury, it could be retained in shallow ponds before discharge. Coincidentally, this should also decrease the concentration of inorganic mercury because of photoreduction of Hg^{2+} to volatile Hg^0 (ref. 9). Knowledge of methylmercury photodegradation would also be used as a criterion for site selection of reservoirs, which commonly contain mercury-contaminated fish. For example, peatland sites are not recommended because water in reservoirs that flood peatlands is stained brown by dissolved organic

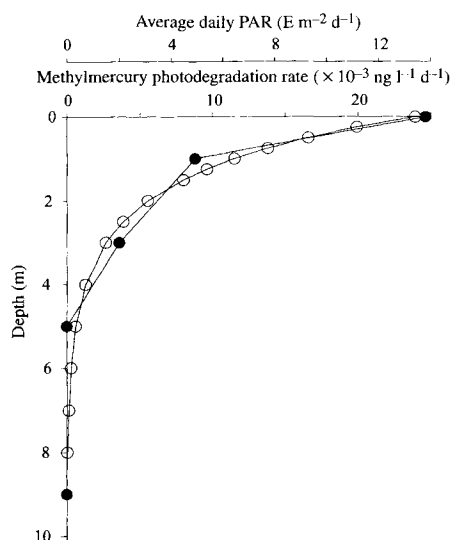


FIG. 3 Depth profiles of photodegradation rate (●) and PAR (○) in Lake 240 (ELA). Same data as in Fig. 2b.

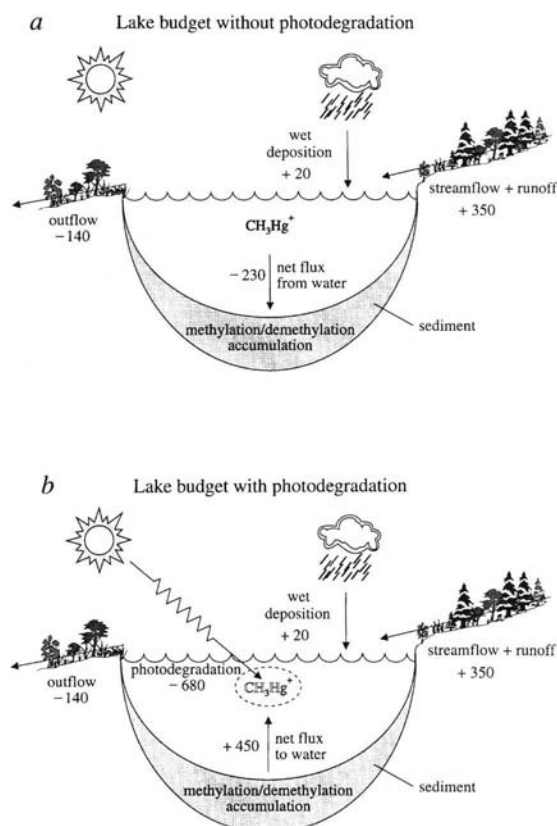


FIG. 4 Preliminary annual mass-balance budget for methylmercury (mg yr^{-1}) in Lake 240 without (a) and with (b) the inclusion of photodegradation in the methylmercury cycle. The net flux of methylmercury to/from the sediments is the net result of methylation, demethylation, and accumulation in the sediments.

METHODS. Direct wet deposition and terrestrial runoff were estimated from previously published data for the ELA²⁰. Stream inflows were calculated from measured volumes and concentrations (our unpublished data). Lake 240 has a surface area of 44.1 ha and for 1994 had an average epilimnetic methylmercury concentration of 0.07 ng l^{-1} and a total mercury concentration of 0.8 ng l^{-1} , an average DOC concentration of $575 \mu\text{mol l}^{-1}$, and an average pH of 7.06. An annual photodegradation rate was calculated as described in text.

matter. This staining reduces light penetration and the beneficial impact of photodegradation.

Our finding that photodegradation is an important sink for methylmercury in lakes has important implications with respect to the understanding of mercury cycling in aquatic ecosystems. For example, a recent study²² (completed before this work) found that external methylmercury inputs to a Swedish lake are equal to the annual accumulation in fish, and consequently it was concluded that in-lake production of methylmercury was unimportant. We should also have concluded that in-lake methylation was unimportant for Lake 240, if we had not known that photodegradation was an important sink for methylmercury. Other recent mass-balance studies have probably also underestimated in-lake methylation rates because methylmercury photodegradation was unknown^{23,24}. The fact that in-lake methylation is more important than previously thought means that attempts to control methylmercury levels in lakes must take into account internal as well as external sources of methylmercury. □

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Active cycling of organic carbon in the central Arctic Ocean

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THE notion of a barren central Arctic Ocean has been accepted since English's pioneering work¹ on drifting ice-islands. The year-round presence of ice, a short photosynthetic season and low temperatures were thought to severely limit biological production^{1,2}, although the paucity of data was often noted. Because primary production appeared to be low^{1,2}, subsequent studies assumed that most organic carbon was either derived from river inputs or imported from adjacent continental-shelf regions^{3,4}. Here we present shipboard measurements of biological production, biomass and organic carbon standing-stocks made during a cruise through the ice covering the central Arctic Ocean. Our results indicate that the central Arctic region is not a biological desert. Although it is less productive than oligotrophic ocean regions not covered by ice, it supports an active biological community which contributes to the cycling of organic carbon through dissolved and particulate pools.

From 26 July to 26 August 1994, the United States and Canada conducted an interdisciplinary expedition aboard USCGC *Polar Sea* and CCGS *Louis S. St Laurent* to study the role of the Arctic Ocean in global change and the potential effects of climate change on biological production in this region. Beginning in the Chukchi Sea, the trans-Arctic station line ran northwards between 170° W and 170° E crossing the Mendeleyev ridge at 80° N, and traversing the Makarov basin. The ships reached the North Pole on 22 August. Two additional stations were completed in the Nansen basin between 84–86° N and 35–38° E before leaving through the Greenland Sea. Here we report microbial and mesozooplankton standing-stocks, primary production, bacterial production and dissolved organic carbon (DOC) concentrations. Sampling was primarily in the upper 100 m of the water column where organic material is most abundant^{2,5–7}.

Surface water entering the Arctic Ocean through the Bering Strait had distinct Pacific Ocean characteristics, readily seen in the initial high Si(OH)₄ concentrations which decreased by a factor of ten along the cruise track (Table 1). Phosphate concentrations decreased by 50% from the Pacific to the Atlantic side, while NO₃⁻ concentrations remained at low to moderate levels in all areas (Table 1). Severe seasonal nutrient depletion commonly observed

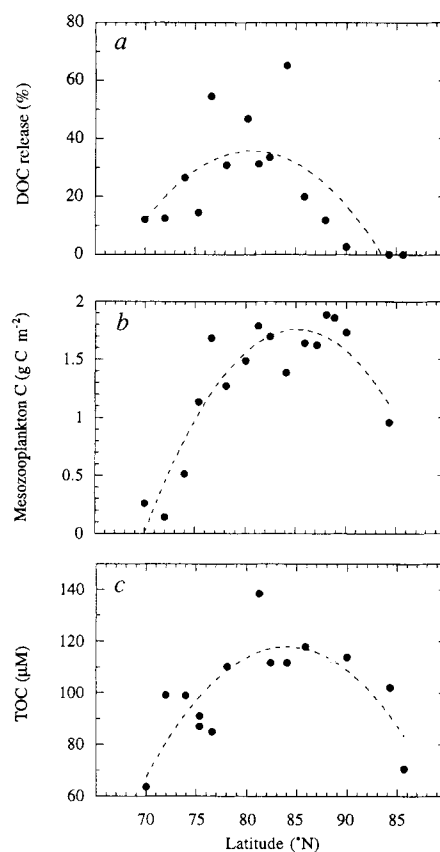


FIG. 1 a, Percentage of total phytoplankton production released as dissolved organic carbon. Phytoplankton here includes any sedimenting ice-algae that are in the water column. Dashed curve, second-order regression with $r^2 = 0.51$. b, Mesozooplankton standing-stocks for 0–100 m depth. Zooplankton were collected in vertical net tows (200- μ m mesh, 0.75-m opening), dried on board ship, weighed and then analysed for carbon with a CHN analyser. Dashed curve, second-order regression with $r^2 = 0.834$. c, Total organic carbon in the surface water. Samples were collected in duplicate, and stored frozen with no preservative. Thawed samples were analysed as described by Benner and Strom³⁰. Dashed curve, second-order regression with $r^2 = 0.634$.

in ice-edge regions⁸ does not seem to extend into most of the central Arctic.

Ice thickness varied from 1.1 to 3.1 m and the ice coverage from 55 to 100%. Lowest ice coverage was observed over the Chukchi shelf (50–80%) and in the Nansen basin (80%), and highest ice coverage was in the Makarov and Amundsen basins (90–100%). Chlorophyll *a* (chl *a*) concentrations in the undersurface of the ice ranged from 0.1 to 297 mg m⁻³, whereas water column chl *a* varied from 0.1 to 5.2 mg m⁻³. Areal concentrations of pigments in the water column were maximal in the Chukchi Sea (162–437 mg m⁻²), decreased to low values (1–22 mg m⁻²) in the Canada, Makarov and Amundsen basins, then increased (60 mg Chl *a* mg m⁻²) in the Nansen basin. Algal biomass in the ice-covered Chukchi Sea was similar to values measured previously⁹ and the maximum concentration in the Nansen basin was similar to values found in the marginal ice zone of Fram Strait¹⁰. In the Arctic basins, areal chl *a* in the water column averaged 12.9 mg m⁻², which is low compared to other oceanic regions¹¹. Ice algal chl *a* ranged from 0.1 to 14.4 mg m⁻² and at times exceeded water-column chl *a*.

Rates of particulate algal production followed the general pattern of pigment distribution. Daily production rates in the water column were as high as 2,670 mg C m⁻² in the Chukchi Sea and as low as 9 mg C m⁻² in the central Arctic, while ice algal productivity ranged from 0.5 to 297 mg C m⁻² d⁻¹. Water-column production decreased in the poleward direction across the Makarov basin, while production by ice algae increased (Table 2). In the Makarov and Amundsen basins, algal production in the ice was about twice that in the water column (Table 2). In the central Arctic, the mean ice algal production was 55 mg C m⁻² d⁻¹ which is similar to values for Arctic first-year ice at lower latitudes^{12,13}. Assuming that the algal growing season is 120 d, our estimate of total annual primary production (water column plus the ice-algae) is ~ 10 g C m⁻², at least one order of magnitude greater than English's estimate¹. The large increase in the estimate of production reflects the importance of ice algae, which were not sampled in previous studies.

Phytoplankton release of DOC varied from 0 to

TABLE 1 Nutrient concentrations in surface water

Latitude	Longitude	Si(OH) ₄ (μM)	NO ₃ ⁻ (μM)	PO ₄ ³⁻ (μM)
70–75° N	169–170° W	20.5 ± 13.3	3.33 ± 3.39	0.92 ± 0.19
76–80° N	173–178° W	11.8 ± 2.3	0.72 ± 0.43	0.92 ± 0.14
81–90° N	31–179° E	6.6 ± 1.6	2.45 ± 0.72	0.74 ± 0.14
84–86° N	35–38° E	2.0 ± 1.6	4.43 ± 1.51	0.46 ± 0.05

Nutrient concentrations (mean ± s.d.) for the upper 20 m of the Arctic Ocean along the transarctic cruise track. Nutrients were analysed on board the ship using standard Technicon Autoanalyzer methods²⁴. Stations are grouped according to depth and geographical region. 70–75° N includes shelf and slope stations in the Chukchi Sea with depths ranging from 40 to 900 m. All remaining stations are in the central basins and depths range from 950 to 4,180 m. 76–80° N in the western sector is in the Canada basin. In the eastern sectors, 81–90° N crosses the Makarov and Amundsen basins, and 84–86° N crosses the Nansen basin.

375 mg C m⁻² d⁻¹ while ice algal release varied from 0 to 44 mg C m⁻² d⁻¹ (Table 2). In the central Arctic, total algal (phytoplankton and ice algae) release rates averaged 36 mg C m⁻² d⁻¹ (Table 2). The percentages of the phytoplankton production released as extracellular carbon were relatively low (12–26%) in the Chukchi Sea, increased significantly (31–65%) in the Canadian basin, then decreased progressively (0–12%) towards the Nansen basin (Fig. 1a). Ice algal release of DOC varied from 0 to 88% with an average value of 34%. Excretion by healthy microalgae is generally less than 30% of the primary production¹⁴. The high DOC release in the Canada basin is probably due to microzooplankton grazing during incubation and/or cell lysis during filtration of water samples.

Particulate organic carbon (POC) collected on glass-fibre filters and measured with an elemental analyser¹⁵ ranged from 3 to 13 μM. Phototrophic carbon estimated from chl *a* (C/chl = 50) accounted for 73% of POC in shelf and slope waters, but only 5–12% of POC in the upper 100 m of deeper waters. Biomass of small heterotrophs (bacterial and protist carbon estimated from microscopic counts¹⁶) accounted for 17% of POC at all stations sampled, while detritus was low at shelf and slope stations, but was 35% of total POC at deeper stations. The dominance of heterotrophic biomass in the central Arctic may result from sampling late in the productive season, but also suggests a potentially high grazing pressure on primary production.

Mesozooplankton in the upper 100 m consisted mostly (87–98%) of copepods, with *Calanus hyperboreus* being most important in numbers and biomass. Previous studies¹⁷ in the Nansen basin indicated a sharp decline in zooplankton standing-stocks north of 83° N. In contrast, we observed increasing zooplankton biomass in the Makarov basin in the northward direction, with a peak at about 87° N (Fig. 1b). Mesozooplankton had normal carbon contents (about 40% dry weight), but their C/N ratios (by weight) were higher (6–8) than in temperate waters¹⁸. High C/N ratios result from low N contents and accumulation of lipids at the end of the productive season¹⁹. Our measurements of mesozooplankton biomass are 2–10 times greater than previous estimates for the central Arctic¹⁹, but similar to biomass reported for the Greenland Sea²⁰. Comparison of the mesozooplankton standing-stock to total POC shows that a large portion (48%) of organic carbon in the central Arctic was present as mesozooplankton biomass during our sampling.

Total organic carbon (TOC) in the surface water ranged from 63 to 139 μM (Fig. 1). As particulate organic carbon usually accounted for about 5–10%

TABLE 2 Chlorophyll concentrations, primary production, and DOC release rates

Latitude	Habitat	Chl <i>a</i> (mg m ⁻²)	Net particulate production (mg C m ⁻² d ⁻¹)	DOC release (mg C m ⁻² d ⁻¹)
70–75° N	Ice	1.6 ± 1.6	31 ± 31	21 ± 21
	Water column	157 ± 99	858 ± 594	125 ± 92
76–80° N	Ice	0.1 ± 0.1	2 ± 1	0.2 ± 0.1
	Water column	11.6 ± 0.3	32 ± 13	21 ± 3
81–90° N	Ice	3.2 ± 2.0	55 ± 41	3.2 ± 2.0
	Water column	7.2 ± 2.7	30 ± 10	24 ± 17
84–86° N	Ice	0.6 ± 0.1	3 ± 1	3 ± 2
	Water column	34.5 ± 25.6	263 ± 238	n.d.

Areal concentrations of chl *a* and primary production (mean ± s.e.) in the Arctic Ocean during July–August 1994. Longitudes are reported in Table 1. Ice habitat includes the bottom of the ice (from ice cores) and the ice–water interface collected by divers. Ice cores were taken with a SIPRE ice corer (7.5 or 20 cm internal diameter) from at least three sites at each station. Light intensity in the open water column was determined with a quantum scalar PAR meter (PNF-300 of Biospherical Instruments Inc.). Light intensity under the ice was determined with a LI-COR LI-190SA underwater quantum PAR sensor. All rates of photosynthesis were calculated for *in situ* light intensity, water-column and sea-ice photosynthesis take into account the relative amount of open versus ice-covered water at each station. Chlorophyll concentrations (determined fluorometrically²⁵) and production (determined by ¹⁴C-bicarbonate²⁶) were corrected for dilution of melted cores with sea water. For the determination of algal biomass and production, the samples were filtered on Whatman GF/F filters. Most incubations lasted ~ 12 h. Release of DOC was measured as described by Zlotnik and Dubinski²⁷. Total net primary production is the sum of the particulate production and the DOC release.

TABLE 3 Comparison of bacterial and primary production and DOC turnover time

Latitude	Bacterial production (mg C m ⁻² d ⁻¹)	Bacterial/primary production	Semi-labile DOC turnover time (yr)
70–75° N	65.9 ± 55.5	0.35 ± 0.40	0.13 ± 0.28
76–80° N	18.7 ± 0.7	0.68 ± 0.45	1.22 ± 0.39
81–90° N	44.7 ± 19.8	1.50 ± 1.49	1.06 ± 0.61
84–86° N	57.7 ± 12.2	0.95 ± 0.80	0.28 ± 0.13

Bacterial production, ratio of bacterial to total primary production, and estimated turnover time of semi-labile DOC in the mixed layer. Bacterial production was measured by a dual-label method with ¹⁴C-leucine and ³H-thymidine²⁸ using open-ocean conversion factors as summarized by Kirchman *et al.*²⁹ Production was calculated from both leucine and thymidine incorporation integrated over the euphotic zone. Total primary production includes the water column and ice algae. Semi-labile DOC was calculated as described by Carlson and Ducklow²³ using the mean value of DOC at 300 m (57 µM) as the refractory pool. Turnover time is given by (semi-labile DOC)/(bacterial production/growth efficiency), where the growth efficiency is 30%.

of TOC, most of this pool consists of DOC. Concentrations of DOC increased in the poleward direction across the Makarov basin and then decreased in the Nansen basin (Fig. 1c). High concentrations of DOC (70–208 µM) in the central Arctic have been attributed to slow rates of degradation², elevated zooplankton activity⁶ and riverine inputs⁷. The major Russian rivers contain high concentrations of DOC²¹, but little information is available on the spatial distribution of this riverine water. Our measurements of substantial primary production and release of DOC indicate that at least a portion of DOC in the central Arctic is produced *in situ*.

Bacterial production ranged from 12 to 152 mg C m⁻² d⁻¹ at the shelf and slope stations and from 15 to 70 mg C m⁻² d⁻¹ in the central basins. These rates are similar to rates reported for open and ice-covered Antarctic waters, respectively²². At times bacterial production equalled or exceeded primary production (Table 3), as reported for Antarctic waters²². Because bacteria are the main consumers of DOC, bacterial production can be used to estimate the mean turnover time of semi-labile DOC (Table 3). Average turnover times ranged from 0.1 to 1.1 years and are similar to turnover times in the equatorial Pacific³³. These relatively high rates of bacterial production and turnover of semi-labile DOC²³ indicate that bacterial activity is not necessarily depressed in cold Arctic waters and that heterotrophic microbial activity plays an important role in carbon cycling.

Our measurements of primary production, bacterial production and zooplankton standing-stocks suggest a dynamic carbon cycle in the surface waters of the central Arctic Ocean. Although production is certainly lower than in shelf regions, the standing-stocks and activity of photosynthetic organisms and heterotrophic consumers across the central Arctic basins indicate a viable biological community. Further studies of biological activity in the central Arctic are clearly needed to determine the relative importance of *in situ* production and consumption of organic carbon compared with allochthonous fluxes of organic material derived from rivers and shelf regions. □

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An Antarctic circumpolar wave in surface pressure, wind, temperature and sea-ice extent

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THE Southern Ocean is the only oceanic domain encircling the globe. It contains the strong eastward flow of the Antarctic Circumpolar Current, and is the unifying link for exchanges of water masses at all depths between the world's major ocean basins¹. As these exchanges are an important control on mean global climate, the Southern Ocean is expected to play an important role in transmitting climate anomalies around the globe. Interannual variability has been often observed at high southern latitudes, and observations of sea-ice extent suggest that such features propagate eastwards around the Southern Ocean^{2,3}. Here we use data from a variety of observational techniques to identify significant interannual variations in the atmospheric pressure at sea level, wind stress, sea surface temperature and sea-ice extent over the Southern Ocean. These anomalies propagate eastward with the circumpolar flow, with a period of 4–5 years and taking 8–10 years to encircle the pole. This system of coupled anomalies, which we call the Antarctic Circumpolar Wave, is likely to play an important role in climate regulation and dynamics both within and beyond the Southern Ocean.

Four types of data were used to reveal the Antarctic Circumpolar Wave (ACW). They are: twice daily atmospheric sea-level pressures (SLP) and meridional wind stresses (MWS) on a 1.875° grid spanning 1985–94 as produced by the European Centre for Medium-Range Weather Forecasts (ECMWF)⁴; monthly averages of sea surface temperatures (SST) on a 2° grid spanning 1982–94 derived from a combination of *in situ* and satellite-radiometer measurements^{5,6}; and daily values of sea-ice concentration on a 25-km grid derived from space-borne microwave sensors spanning 1979–91 (ref. 7). Northward sea-ice extents (SIE) are determined at increments of 5° longitude by the 15%

concentration threshold⁸. Time series of monthly anomalies in each parameter are determined relative to the applicable record-length monthly mean values, thus removing the average seasonal cycles. To suppress further seasonal and possible biennial signals, and to remove long-term trends, all time series are band-passed with a filter⁹ having a 3–7 year admittance window. As we show, this filtering extracts the most important interannual variability occurring in all the records. The sign convention is that positive values denote higher-than-normal SLP, northward MWS anomalies, warm SST anomalies, and northward SIE anomalies.

Time–longitude diagrams of anomalies in the four parameters (Fig. 1 top, along 56°S for SLP, MWS and SST) show clear patterns of dominating eastward propagation. The ranges are as large as 8 mbar in SLP, 0.3 dyn cm^{-2} in MWS, 1.6°C in SST and 350 km in SIE. They appear to be most intense in the Pacific sector, and in each parameter they take the general form of two wavelengths that end-to-end encircle the globe. Though particular phases are sometimes interrupted, the overall ACW pattern progresses eastward at average speeds of $6\text{--}8 \text{ cm s}^{-1}$, taking 8–10 years for individual phases to travel around the globe. Variability at particular locations thus occurs on timescales of 4–5 years. Two-dimensional auto-spectra computed from the unfiltered monthly anomalies (Fig. 1 bottom) verify this, and they clearly show these to be the most energetic variations occurring over interannual timescales. This is also consistent with the ACW being observed contemporaneously with satellite altimeter measurements of sea level height (G. Jacobs and J. Mitchell, personal communication).

A feature to note in Fig. 1 is the strong poleward anomaly in SIE near 90°W that persisted from 1988 to 1991. This is the extraordinary retreat in sea ice reported for that period in a region west of the Antarctic Peninsula (Bellingshausen Sea)¹⁰, which was especially intense during the summer months and which was preceded by two years of exceptionally heavy ice conditions (near the end of the retreat, winter ice cover returned to normal-to-above-normal conditions¹¹, but the summer retreat still dominated). It is apparent that these local fluctuations in sea ice were manifestations of a propagating set of coupled anomalies that can be traced back towards the west to the Indian Ocean some 5 years earlier and which subsequently continued on through Drake Passage and farther east with the circumpolar flow. A long record of fast-ice (sea ice attached either to land or to land-bound ice) duration in the South Orkney Islands (60.5°S , 45.5°W) has recently been analysed and related to the hemispheric ice field³. The observed local interannual variability agrees well with the propagation of the ACW shown in Fig. 1, while autocorrelations of hemispheric ice data indicate an eastward propagation of interannual anomalies of $30^\circ\text{--}60^\circ$ longitude per year³ consistent with the present phase speeds of $\sim 40^\circ$ per year. Interannual

variability in ice cover is critical to local biology, such as krill and salp populations¹², but because these anomalies propagate and are quasi-symmetric about the pole, only a weak signal in total ice cover on these timescales¹³ results.

Lagged cross-correlations of SST anomalies versus the other three parameters (Fig. 2) show warm (cold) SSTs trailing high (low) SLPs by 1 year ($\sim 90^\circ$) and approximately 180° out of phase with equatorward (poleward) anomalies in MWS and SIE. These phase relationships are shown schematically in Fig. 3, which also shows the course of the Antarctic Circumpolar Current (ACC) as represented by an average of its two principal fronts¹⁴, as well as interactions between the ACW and the more northerly subtropical domains.

The ACW anomalies in SST appear to originate in the western subtropical South Pacific, and spread south and east into the Southern Ocean, where subsequent eastward propagation is due mainly to the ACC. This is demonstrated in Fig. 4. The top panel

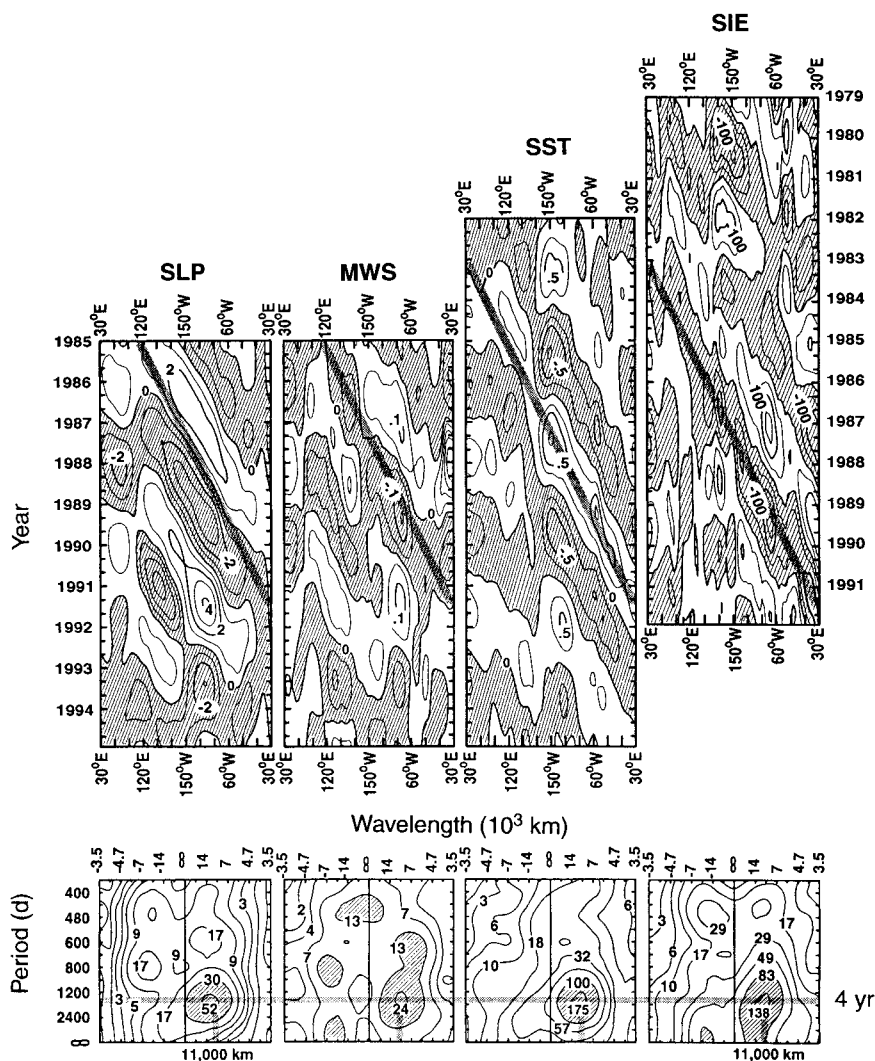


FIG. 1 Top panels, time–longitude diagrams of interannual anomalies along 56°S in sea-level pressure (SLP), meridional wind stress (MWS), sea surface temperature (SST) and, along 5° longitude increments, in meridional sea-ice extent (SIE). Negative and southward anomalies are shaded. The grey bars in all panels are synchronous in time and space. Contour intervals are 1.0 mbar for SLP, 0.05 dyn cm^{-2} for MWS, 0.25°C for SST and 50 km for SIE. Bottom panels, two-dimensional auto-spectra of the above quantities, computed from unsmoothed monthly anomalies. Positive wavelengths denote eastward propagation. Contoured values are in power units of the above quantities (units-squared times frequency), and are selected such that each contour is statistically independent from its neighbours.

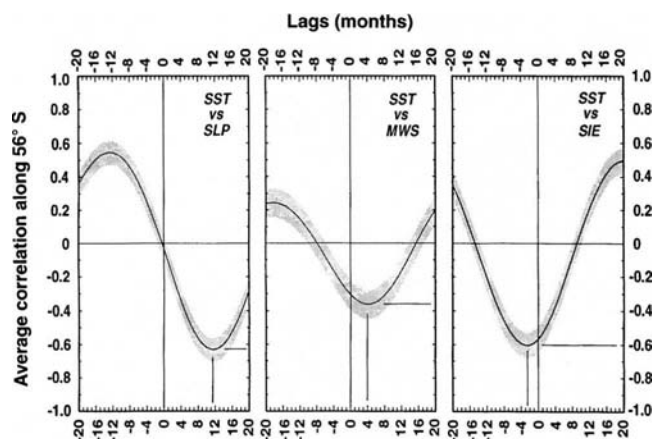


FIG. 2 Lagged cross-correlations of the indicated interannual quantities, averaged along 56°S. 95% confidence intervals are indicated by grey shading (calculated according to Fischer's Z-transform). Positive lags indicate the first quantity leading the second, and vice versa for negative lags.

shows surface speeds, computed from historical hydrographic data that were objectively smoothed and mapped onto a 1° grid¹⁵, relative to a pressure level of 1,000 dbar (~1,000 m depth) where the mean currents are presumed to be weak. These data do not resolve the narrow frontal jets where surface speeds often exceed 40 cm s⁻¹, and where, in Drake Passage the jets comprise only ~20% of the width of the current¹⁶. Elsewhere they probably account for even less. The gridded data provide information about speeds averaged over several degrees latitude, and in the Southern Ocean these speeds, ~3–15 cm s⁻¹, bracket the propagation speeds of the ACW. Earlier studies show that

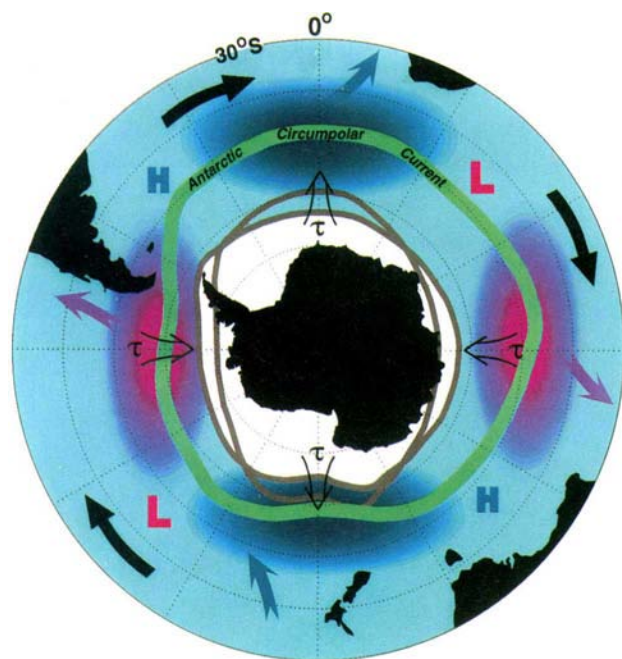


FIG. 3 Simplified schematic summary of interannual variations in sea surface temperature (red, warm; blue, cold), atmospheric sea-level pressure (bold H and L), meridional wind stress (denoted by τ), and sea-ice extent (grey lines), together with the mean course of the Antarctic Circumpolar Current (green line)¹⁴. Sea-ice extent is based on the overall 13-year average in our data. Heavy black arrows depict the general eastward motion of anomalies, and other arrows indicate communications between the circumpolar current and the more northerly subtropical gyres.

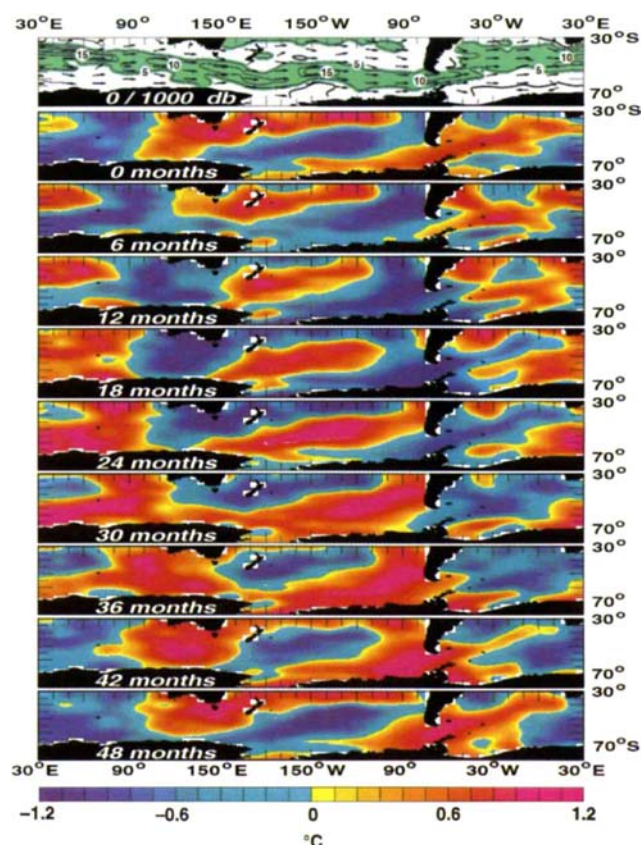


FIG. 4 Top panel, surface geostrophic velocity vectors relative to 1,000 dbar (~1,000 m depth) calculated from new atlas data¹⁵. Eastward components of ≥ 5 cm s⁻¹ are shaded. Lower nine panels: lag-sequence of the dominant mode of an extended empirical orthogonal function analysis^{18,19} of interannual anomalies of sea surface temperature (SST), which in this case accounts for 51% of the total SST variance for the ten-year period of 1985–94. The extended empirical orthogonal function is computed from the covariance of state vector for standardized interannual SST anomalies, yielding estimates with equal variance over the field. This allows propagation to be seen unbiased by regions of strong and weak variance. Contour intervals for the lag-sequence of SST is given by the colour bar, ranging over ± 1.2 °C.

interannual SST anomalies can in fact be carried along by the mean ocean currents¹⁷.

The lower panels in Fig. 4 show a time-lag sequence of the dominant mode of an extended empirical orthogonal function analysis^{18,19} conducted with the interannual SST anomalies. This analysis reveals the most important patterns of propagation contained in the data. The lag-sequence in the figure spans 4 years, or half a complete two-wave cycle. Initially, warm anomalies are seen south of Australia and within Drake Passage, with intervening cold anomalies. The warm anomaly south of Australia stretches northeastwards across most of the subtropical South Pacific, and in following lags it compresses near middle latitudes and drifts southeast into the Southern Ocean. Part of it turns north along the coast of Chile with the Peru (Humboldt) Current, whereas at the end of the sequence the remainder passes through Drake Passage, having travelled over $\sim 180^\circ$ of longitude during the four years. This general pattern of propagation through the Pacific is also apparent with cold anomalies.

The SST anomalies follow the hemispheric course of the ACC, but there is also a superimposed northward component of propagation in most regions that is probably due to Ekman layer flow from the prevailing eastward winds, and this facilitates a spreading of anomalies associated with the ACW northward into the subtropics. As with portions of the anomalies branching off into the

southern Peru Current, portions likewise enter the southern Benguela Current in the eastern South Atlantic and spread equatorwards in the South Indian Ocean, though largely outside the bounds of the illustrations. Only in the western Pacific do anomalies clearly enter the Southern Ocean from the north.

The initiation of the ACW is probably associated with El Niño activity in the equatorial Pacific, possibly through an atmospheric teleconnection with higher southern latitudes^{20–22}. The south-eastward propagation of anomalies in the western Pacific needs to be studied, as do their subsequent movements around the Southern Ocean and into the three major basins to the north. Clearly some mechanisms must exist to maintain the ACW against dissipation as it travels around the globe; the ocean, atmosphere and ice field must be coupled so as to effect net positive feedbacks. Such feedbacks can be expected to alter the propagation of the ACW, leading to a state that is dynamically richer than would be expected from simple advection alone. □

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Histology of the first fish

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THE first description of *Anatolepis* Bockelie & Fortey was from early Ordovician sediments of Ny Friesland, Spitsbergen^{1,2}, but the genus is now known from many localities in North America and Greenland, ranging in age from the Late Cambrian period to the Early Ordovician^{3–6}. Although initially interpreted as an agnathan fish^{2,3} that predated other representatives⁷, this has been widely disputed because the available histological data were unconvincing^{6,8–10} and the scales fell outside the known morphological range of other accepted early vertebrates^{9–11}. Further doubt was cast upon the vertebrate affinity of *Anatolepis* when specimens from East Greenland were interpreted as the cuticular fragments of aglaspoid arthropods⁶, although this interpretation has also been refuted¹². Here we report on the morphology and histology of large collections of *Anatolepis*, and demonstrate the

presence of dentine, a tissue unique to vertebrates, confirming that the taxon is both a vertebrate and the oldest known fish.

To resolve the taxonomic position of *Anatolepis*, all available material was examined, including Cambrian material from Wyoming, New York, Utah and Oklahoma. Published illustrations of *Anatolepis* show clear variation in the ornamentation and size of the sclerites, and this has been used to tentatively accept some specimens assigned to the genus but refute others¹³. From the entire collections, however, it is clear that there is only a single species of *Anatolepis*, *A. heintzi* Bockelie & Fortey², with a clear overlap of ornamentation patterns and tubercle size. The tubercles range from circular to ovate, and the sheets of phosphatic tissue between the scales vary from flat to strongly curved (Fig. 1). Although the species has been documented from several localities distributed around the margin of Laurentia^{1–6}, the amount of material recovered is small, and in total amounts to a few square centimetres of exoskeleton. There is therefore a limit to the amount of morphological data that can be retrieved from these fragments, and the histology of the hard tissues offers one of the few means of clarifying the relationships of *Anatolepis*.

Histologically, sclerites of *Anatolepis* are divisible into two

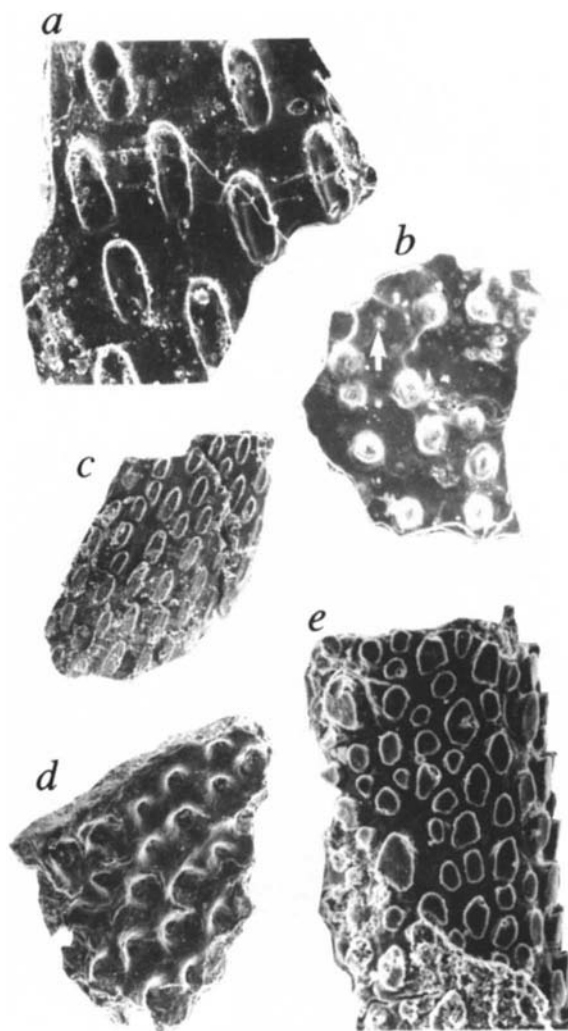


FIG. 1 a–e, Representative examples of *Anatolepis heintzi* Bockelie & Fortey from the Late Cambrian Deadwood Formation, Crook County, Wyoming, USA. All are upper views except d, which is basal. a, USNM 488566, magnification $\times 95$. b, Arrow indicates position of one of the pore canals within the lamellar tissue; USNM 488567, magnification $\times 95$. c, USNM 488568, magnification $\times 32$. d, USNM 488569, magnification $\times 32$. e, USNM 488570, magnification $\times 158$. All material is deposited in the National Museum of Natural History, Washington DC, USA.

tissues. Tubular dentine forms the individual tubercles, with each tubercle containing a single pulp cavity that opens on the under-surface (Figs 1d and 2). Dentine tubules radiate from the tip of the pulp cavity and, at around mid-height, simple, branching tubules of 0.5–1 μm diameter extend towards the crown surface (Fig. 2). Some tubules show marked narrowing (dt in Fig. 2b), and finer tubules close to the crown surface provide evidence of a terminal

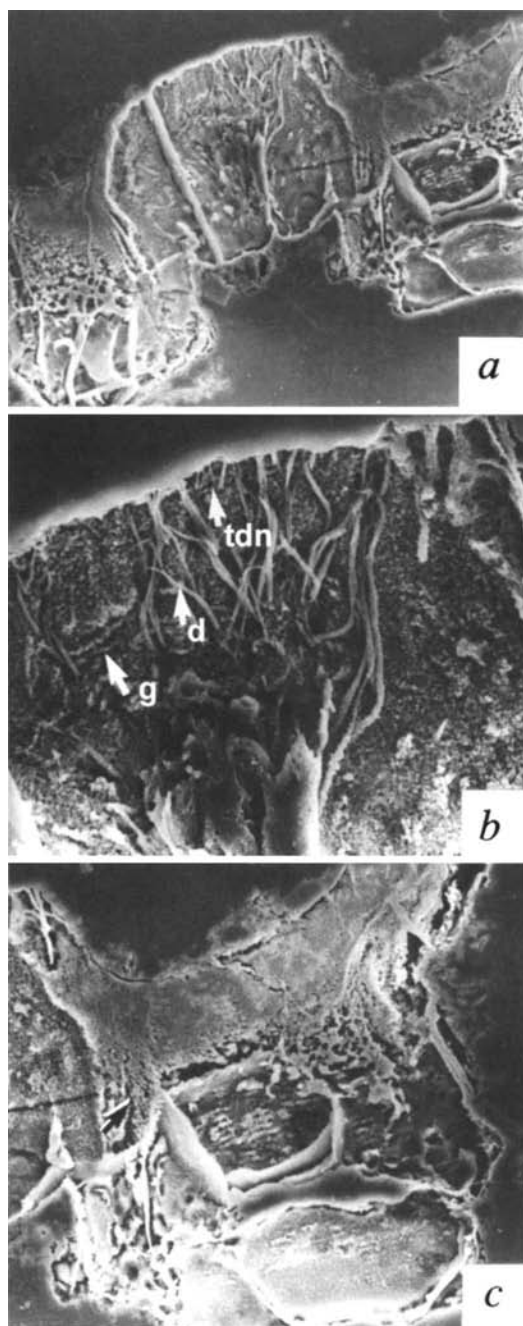


FIG. 2 a–c, Scanning electron microscopy images of *Anatolepis heintzi* Bockelie & Fortey in longitudinal section, USNM 488571. a, Tubercle flanked by lamellar material on either side, magnification $\times 410$. b, Close-up of tubercle showing pulp cavity and radiating dentine tubules. The tubules may end at the crown surface in a terminal dentine network, magnification $\times 1,335$. Abbreviations: dt, narrowing dentine tubule; gl, incremental growth lines; tdn, possible terminal dentine network. c, Lamellar tissue lying between individual tubercles, magnification $\times 680$. Note the concave edges of the lamellae at the junction with the tubercles (arrowed in c).

network (tdn in Fig. 2b). The tubules do not penetrate to the crown surface of the tubercle, as there is no sign of emergent tubules on external, crown surfaces of the tubercles in the three-dimensional specimens. Incremental lines can be discerned within the dentine in some specimens (gl in Fig. 2b). There is no evidence for a capping tissue to the odontodes in the material we have examined to date. The odontodes are surrounded by a different, microcrystalline lamellar tissue with lamellae 0.5–1 μm thick. Pore canals seem to penetrate the full thickness of this tissue (Figs 1b and 3), in contrast to the dentine. The lamellae are strongly deflected upwards around the junction with the odontodes and the pore canals, indicating centrifugal growth (Figs 2c, 3b and 4). An arthropod affinity for *Anatolepis* can be confidently discounted, because arthropods do not have tubules/pore canals that radiate from a large central cavity. The pore canals or dermal ducts of arthropods are single, discrete structures lying perpendicular to the cuticular surface. They penetrate the whole thickness of the carapace, and often terminate in funnel-shaped apertures on the inner and outer surface of the cuticle^{12,14–16}.

Although the individual odontodes are formed from a clearly recognizable dentine, the interconnecting lamellar tissue has no obvious counterparts amongst other Palaeozoic vertebrates. Curiously, hyaloine described from recent armoured catfish (Siluriformes, Callichthyidae)¹⁷ bears several similarities with this tissue. Hyaloine is a highly mineralized tissue that resembles enameloid in some respects and, to a lesser degree, ganoine. It differs in not overlying dentine, in being less mineralized than these tissues, and in being deposited on the scute surface at some distance from the epidermis, principally from osteoblasts. Hyaloine has also been identified on the free surface of elasmoid scales in some teleosts and in some lizards¹⁷. It is not yet clear, however, whether the resemblance of the lamellar tissue in *Anatolepis* to hyaloine represents a histogenic commonality or a superficial resemblance.

Dentine is a tissue unique to vertebrates, and its presence in

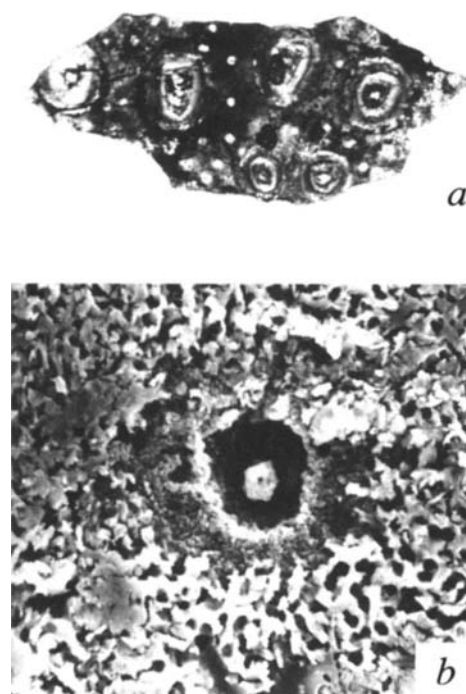


FIG. 3 a, Transmitted light image of *Anatolepis heintzi*, USNM 488572 in transverse section showing the pore canals (pale circles between larger tubercles) which penetrate the lamellar intertubercular areas, magnification $\times 60$. b, SEM image of an etched transverse section of a pore canal showing lining caused by deflection of the lamellar tissue; same specimen as a, magnification $\times 1,800$.

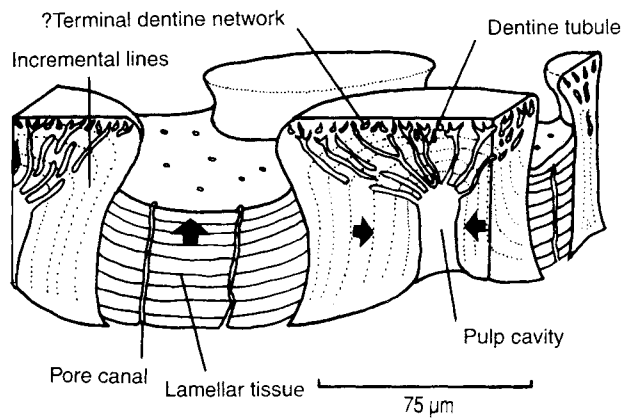


FIG. 4 Block reconstruction of hard tissues and structures within *Anatolepis heintzi*. Arrows indicate directions of growth.

Anatolepis together with pulp cavities and a possible terminal dentine network provide the first definitive statement regarding the affinity of the taxon. The demonstration that *Anatolepis* is a vertebrate has numerous implications for studies of early vertebrate phylogeny. (1) Specimens of *Anatolepis* lack a honeycombed or cavernous layer (*contra* Repetski⁴), a condition often cited as primitive for vertebrates^{13,18,19}. (2) The occurrence in *Anatolepis* of individual odontodes linked by a sheet of lamellar tissue may be a condition from which both micro- and macromeric exoskeletons could have evolved. Loss of the lamellar tissue would yield a micromeric exoskeleton, whereas increased biomineralization around and below the individual odontodes could give rise to macromeric plates. (3) The absence of plates with clearly defined margins suggests that the hard tissues of *Anatolepis* comprised an exoskeletal covering of the anterior of the body, and that the tail was unmineralized. (4) *Anatolepis* first appeared at about the same time as the earliest known euconodonts at the base of the Trempealeau stage (latest Cambrian). With the affirmation of euconodonts as vertebrates^{20–23}, the addition of *Anatolepis* to the group indicates that the initial evolutionary radiation of the vertebrates was already underway by the Late Cambrian. (5) The morphology and histology of *Anatolepis* do not suggest a close relationship with any of the Cambro-Ordovician vertebrates described to date. Recent studies of microvertebrate remains from the Ordovician of Colorado^{24,25}, Australia (G. Young, personal communication), South America²⁶ and Siberia²⁷ have yielded sharks, thelodonts, osteostracans, possible acanthodians and heterostracomorphs. The recognition of *Anatolepis* as a vertebrate adds a further lineage to this phylogeny, demonstrating that the Late Cambrian–Ordovician is one of the principal evolutionary episodes in the history of fish. □

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Insect species diversity, abundance and body size relationships

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BIOLOGICAL diversity, population size and body size are interdependent^{1–8}, but there is little consensus on the nature or causes of these relations. Here we analyse the most thoroughly sampled ecological community to date, a grassland insect community sample containing 89,596 individuals of 1,167 species. Each taxonomic order had a distinct body size at which both species richness and number of individuals were highest, but these peak sizes varied more than 100-fold among five major orders. These results suggest that there may be fewer undiscovered small insect species than previously thought. Moreover, we found a surprisingly strong, simple, but unreported, relation between species richness (S) and the number of individuals (I) within size classes, $S = I^{0.5}$. Because this held across numerous body types and a 100,000-fold body-size range, there may be a general rule that is independent of body size for the relations among interspecific resource division, abundance and diversity.

Local diversity comes from the balance between immigration of new species and local extinction⁹. Immigration rate depends on the number of species already present⁹ and dispersal ability, which is likely to be dependent on body size¹⁰. Extinction rate depends on population abundances and their distribution, with rare species more likely to become extinct as a result of population fluctuations¹¹. Because of this, species richness (S) should depend on the number of individuals (I) within a group of interacting species, here assumed to be species of similar body size, especially within taxonomic orders^{12,13}. The number of rare species also depends on the abundance distribution (for example, log-normal¹⁴, broken-stick¹⁵), as will the precise relation between S and I .

Assuming a minimum population size for population persistence, and abundances following a broken-stick model¹⁵, it has been shown¹⁶ that $S = cI^{0.5}$, where c is a case-specific constant. The relation $S = cI^{0.25}$ has previously been derived¹⁴ for a canonical log-normal distribution. For our data we investigate the effect on species richness of the total number of individuals in each size class¹⁷. We also examine the relations among species richness, number of individuals, and body size.

We sampled insects 7–9 times throughout the 1992 growing season in each of 48 grassland fields and savannahs at Cedar Creek, Minnesota, and determined the abundance and computed the biovolume of each species (for methods see Fig. 1 legend). These data are especially powerful because they include numer-

ous simultaneously sampled taxonomic orders spanning a broad range of body sizes.

For our entire fauna, peak species richness occurred at the intermediate body size that led to the maximum number of individuals (Fig. 1A, B). The same pattern occurred within each of the five most abundant orders, although peak body sizes differed (Fig. 2A, B). Decreases in numbers of individuals at small body sizes within each order were not caused by size-biased sampling because these decreases occurred in all orders,

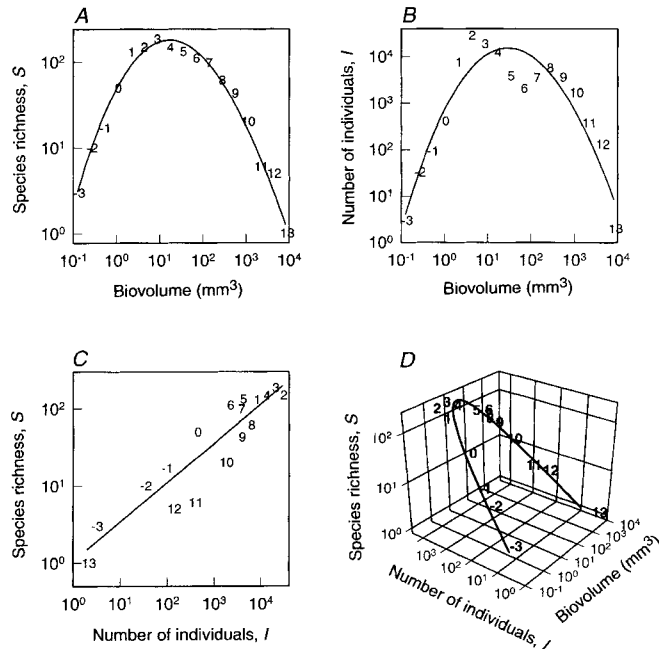


FIG. 1 A, Relation between species richness (S) per $\log_2$ biovolume class and biovolume (B) fitted by the extreme value function $\ln S = a + b \exp(-\exp\{-(\ln B - c)/d\}) - [(\ln B - c)/d] + 1$, an asymmetric peak function; $a = -55.3$, $b = 60.5$, $c = 2.81$, $d = 13.9$, $r^2 = 0.98$, $N = 17$, $P < 0.01$. Note that numbers shown identify 2^{number} mm³ size classes (for example, the -1 shows where organisms of size 2^{-1} mm³, or $1/2$ mm³, fell on the graph). B, Relation between number of individuals (I) within $\log_2$ biovolume classes and biovolume with fitted parameters of $a = 284.6$, $b = 294.3$, $c = 3.37$, $d = 23.8$, $r^2 = 0.88$, $N = 17$, $P < 0.01$. C, Relation between species richness (S) and number of individuals (I) within $\log_2$ biovolume size classes, fitted by regression with $S = 1.05 I^{0.51}$, $r^2 = 0.85$, $N = 17$, $P < 0.01$. For the five most abundant orders: Coleoptera, $S = 1.03 I^{0.48}$, $r^2 = 0.95$, $N = 10$, $P < 0.01$; Diptera, $S = 1.21 I^{0.57}$, $r^2 = 0.94$, $N = 8$, $P < 0.01$; Hemiptera, $S = 1.28 I^{0.35}$, $r^2 = 0.91$, $N = 11$, $P < 0.01$; Hymenoptera, $S = 1.10 I^{0.63}$, $r^2 = 0.91$, $N = 14$, $P < 0.01$; and Orthoptera, $S = 1.14 I^{0.26}$, $r^2 = 0.63$, $N = 7$, $P < 0.05$. D, Relation between species richness and number of individuals within $\log_2$ biovolume classes and biovolume using fitted curves from A and B.

METHODS. Insects were sweep sampled from 48 grassland fields and savannahs at Cedar Creek, Minnesota, 7–9 times each throughout the 1992 growing season, with 100 sweeps per sample. In total, 89,596 individuals of 1,167 species were captured and enumerated. Sweep-net samples give excellent estimates of relative abundance within orders²⁹, and good estimates of relative abundance between orders³⁰. Our conclusions require only that measures of abundance be relative. All specimens were manually sorted and identified to species (89.8% of specimens) or morphospecies within known genera or families. Species biovolume, an index of biomass, was calculated as the average product of length (from tip of head to tip of abdomen), width (maximum width of body or head) and thickness (maximum thickness of body or head) for 5 specimens in the oldest life stage for each species, even though body size increases greatly during development. Most species (84%) only occurred as adults in our samples. To minimize counting a species twice, all larvae were examined by outside experts and, if they might have been the same species as an adult insect, we counted them as a single species.

even though peak body sizes differed 100-fold. Estimates of total species richness from species accumulation curves (Fig. 3) gave results almost identical to those using observed species richness (S). Thus differences in species richness among size classes were real, and not artefacts of sampling effort^{18,19}.

For our entire fauna, the dependence of S on I was best fit by $S = 1.05 I^{0.51}$ (Fig. 1C; $r^2 = 0.85$, $N = 17$, $P < 0.01$). Similar relations occurred in the five most abundant orders (see Fig. 1 legend). A size class with 10 times as many individuals had, on average, 3.2 times as many species. Smaller-bodied size classes had slightly more equitable abundance distributions, and slightly but consistently more species from the same number of individuals (Fig. 1C). Examined in three dimensions, S , I and biovolume formed a parabola (Fig. 1D), which collapsed to the $S = 1.05 I^{0.51}$ line when projected onto the S and I plane. Because processes such as competition, predation, herbivory and dispersal are size depen-

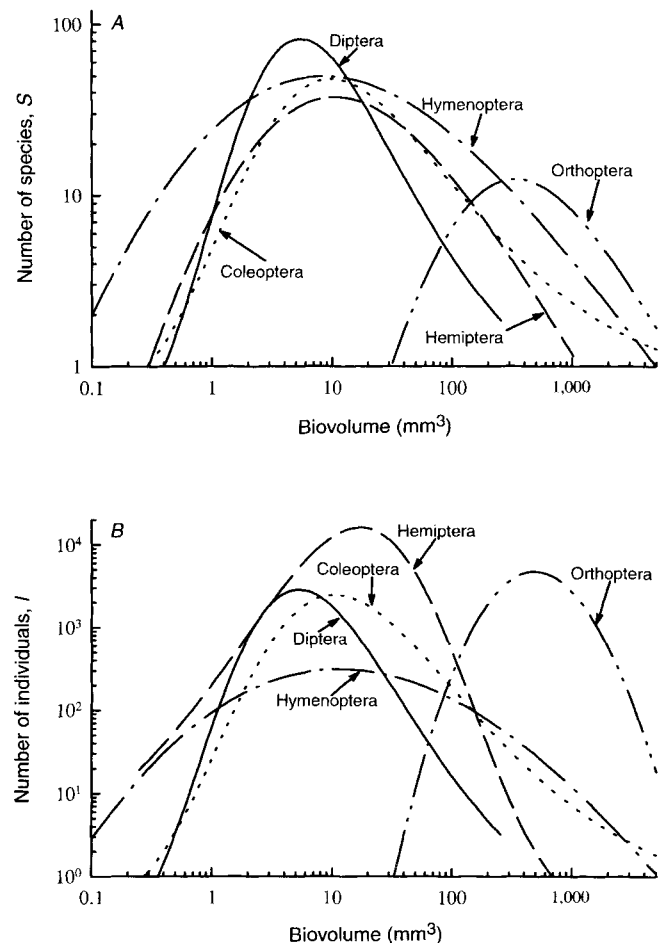


FIG. 2 A, The relation between species richness in $\log_2$ biovolume classes and biovolume for the five most abundant and speciose orders in our samples. The curve for each order is from the fitted extreme value function (Fig. 1). Coleoptera, $a = -0.44$, $b = 4.3$, $c = 2.27$, $d = 2.22$, $r^2 = 0.91$, $N = 10$, $P < 0.01$; Diptera, $a = -0.99$, $b = 5.4$, $c = 1.69$, $d = 1.8$, $r^2 = 0.93$, $N = 8$, $P < 0.01$; Hemiptera, $a = -8.8$, $b = 12.5$, $c = 2.35$, $d = 3.13$, $r^2 = 0.97$, $N = 11$, $P < 0.01$; Hymenoptera, $a = -10.8$, $b = 14.6$, $c = 2.13$, $d = 7.0$, $r^2 = 0.96$, $N = 14$, $P < 0.01$; Orthoptera, $a = -5.6$, $b = 8.12$, $c = 5.80$, $d = 3.13$, $r^2 = 0.99$, $N = 7$, $P < 0.01$. B, The relations between number of individuals of all species in each $\log_2$ biovolume class and biovolume. Coleoptera, $a = -0.99$, $b = 8.8$, $c = 2.35$, $d = 2.3$, $r^2 = 0.88$, $N = 10$, $P < 0.01$; Diptera, $a = -2.32$, $b = 10.3$, $c = 1.66$, $d = 2.0$, $r^2 = 0.93$, $N = 8$, $P < 0.01$; Hemiptera, $a = -0.94$, $b = 10.6$, $c = 2.82$, $d = -2.3$, $r^2 = 0.84$, $N = 11$, $P < 0.01$; Hymenoptera, $a = -49.9$, $b = 55.7$, $c = 2.44$, $d = 12.1$, $r^2 = 0.89$, $N = 14$, $P < 0.01$; Orthoptera, $a = 306.2$, $b = 314.5$, $c = 6.16$, $d = 12.0$, $r^2 = 0.96$, $N = 7$, $P < 0.01$.

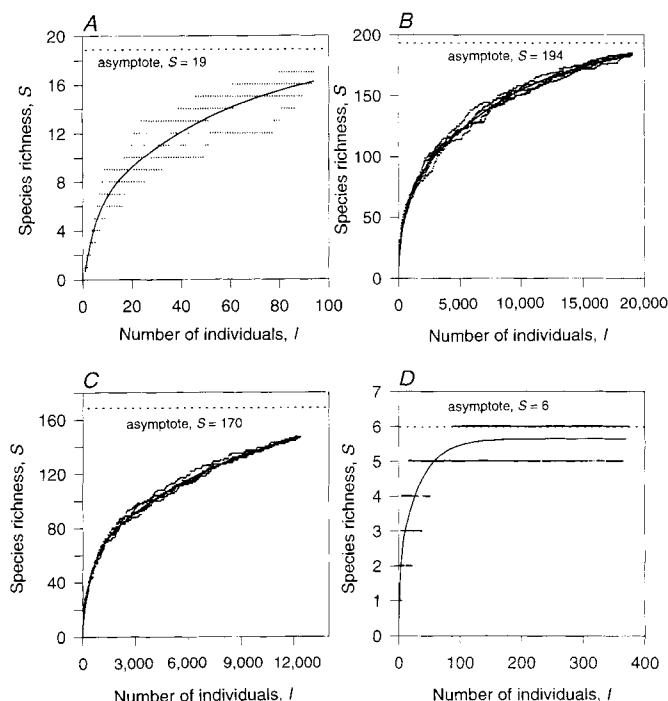


FIG. 3 Species accumulation curves¹⁸ show the effect of sampling effort on observed species richness. For each size class, increasingly larger random subsamples from the set of all individuals of all species in the size class were drawn to construct a species accumulation curve, with a maximum of 500 such random draws per curve. The resulting curve for each size class was fitted with an asymptotic, negative exponential function to estimate the asymptote, which is the number of species estimated with infinite sampling effort¹⁸. Only the smallest size class had no asymptote. The relation of asymptotic species richness (S_{asympt}) and number of individuals (I) was virtually identical to the relation of S and I ; $S_{\text{asympt}} = 0.95 I^{0.53}$, $r^2 = 0.80$, $N = 16$, $P < 0.01$. The accumulation curves for four size classes are shown. A, Size class -1 (0.25–0.5 mm³); B, size class 3 (4–8 mm³); C, size class 4 (8–16 mm³); D, size class 11 (1,024 to 2,048 mm³).

dent^{3,6,10}, it is surprising that the relation of numbers of individuals to species richness is not dependent on body size.

Intriguingly, our results are consistent with May's¹⁶ approximation $S = cI^{0.5}$, but our abundance distributions within size classes were not broken-stick. Rather, they followed the power function $A_r = A_1/r^m$, reported for whole communities^{1,8}, where A_r is the abundance of the r th most abundant species in the size class; for our data, $m = 1.94$. It can be shown (E.S. *et al.*, manuscript in preparation) that $S = cI^{1/m}$ if size classes have power-function abundance distributions with equal m , if all size classes have the same minimum population size below which extinction occurs, and if the division of resources within each size class is inequitable ($m > 1.5$). This gives $S \sim I^{1/1.94} = I^{0.5}$. Thus $S = I^{0.5}$ suggests that the same rules determine diversity and abundance across a 100,000-fold range in insect body sizes.

Most studies have found unimodal relations between species richness and body size^{2,3,6,20,21}, and between number of individuals and body size^{1,3,8,22–24}. Although hypotheses incorporating trade-offs, for example between metabolic efficiency and reproductive rate^{25,26}, predict unimodal relations between species richness and body size, none predicts that the number of individuals should peak at the same intermediate size. The differences among modal sizes of the five insect orders suggest that shared physiologies and/

or morphologies from the common genetic heritage of an order limit related organisms to similar sizes.

Global diversity based on currently described species is a unimodal function of body size⁶. May⁶ proposed that actual global diversity may be highest at the smallest sizes, with small species fantastically undersampled, representing many millions of undescribed species. Our data suggest that undescribed species are more likely to be of intermediate sizes within any taxonomic group, which is supported by studies of tropical forest canopy beetles^{1,8,20}. This seems to support May's alternative hypothesis that diversity peaks at some intermediate body size⁶, and would seem to suggest that global diversity is closer to the lower end of the estimate 10–50 million species⁷. However, if $S = I^{0.5}$ holds for nematodes, bacteria and viruses, it would suggest these phenomenally abundant small-bodied taxa might constitute most of the Earth's diversity, giving a total global diversity towards the higher end of May's estimate. Any such extrapolations are speculative until we know if the patterns we report for insects hold for other groups, and how the geographical turnover of species depends on body size^{27,28}. Such relationships, once observed in other communities, would provide vital clues to the diversity of life on Earth, and the causes of this diversity. □

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High levels of genetic change in rodents of Chernobyl

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BASE-PAIR substitution rates for the mitochondrial cytochrome *b* gene of free-living, native populations of voles collected next to reactor 4 at Chernobyl, Ukraine, were estimated by two independent methods to be in excess of 10^{-4} nucleotides per site per generation. These estimates are hundreds of times greater than those typically found in mitochondria of vertebrates, suggesting that the environment resulting from this nuclear power plant disaster is having a measurable genetic impact on the organisms of that region. Despite these DNA changes, vole populations thrive and reproduce in the radioactive regions around the Chernobyl reactor.

The meltdown of reactor 4 on 26 April 1986 at the Chernobyl nuclear power plant was estimated to have released 50–200 million curies of radiation^{1–3} plus a variety of chemical and metal pollutants. To estimate the biological significance of this accident, we have compared the genetic variation of voles living at the Chernobyl site (Chernobyl samples) exposed to this pollution^{3,4} with those collected from a site that received minor amounts of Chernobyl-related pollution (control samples)⁴. The 1,143-base-pair (bp) cytochrome *b* gene was sequenced^{5,6} from two species of voles (*Microtus arvalis*, $n = 5$, Chernobyl sample; $n = 5$, control region; and *M. rossiaemeridionalis*, $n = 4$, Chernobyl sample; $n = 5$ control region). The Chernobyl sample was collected 1 km southwest of reactor 4 in an area formerly known as the Red Forest (UTM 36, 306204E, U 5683716N (coordinates give kilometers east of the 36th TransMercator line and north of equator) 51° 16' 18.4" N, 30° 13' 18.0" E), whereas the control sample was collected from west of Stracholossye, 32 km southeast of reactor 4 (UTM 36, 318000E, U 5661778N; 51° 04' 43.0" N, 30° 24' 06.1" E)⁷. No morphological abnormalities were noted in dissections and specimen preparation of any of these rodents, although eight of 191 specimens from Chernobyl had spleens enlarged to up to six times the normal size⁷.

Using phylogenetic methods (parsimony), the minimum number of nucleotide changes was calculated^{8,9} for the control

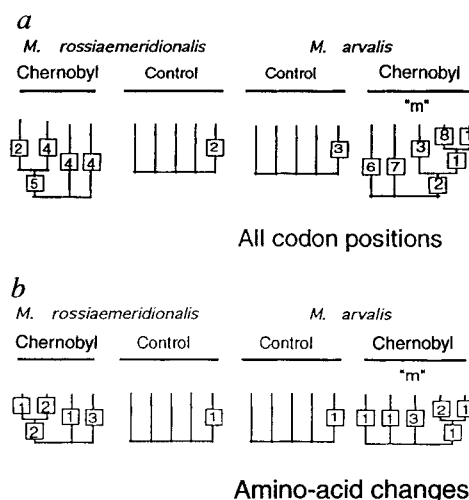


FIG. 1 Gene trees produced by parsimoniously arranging the minimum number of genetic changes (numbers shown in boxes) within experimental and control populations of two species of voles⁷. *a*, Tree based on all codon positions; *b*, tree based on amino-acid changes only. At least two independent clones for all individuals were amplified, cloned and sequenced in both directions. Each sequence was scored blind relative to the opposing strand and other clones; 80% of individuals were scored without knowledge of control or experimental status. DNA sequence data were used as discrete, unordered phylogenetic characters, and trees were constructed using version 3.0s of PAUP²³. Minimal numbers of mutations and character-state changes occurring on each lineage were evaluated using PAUP²³ and MacClade²⁴. Amino-acid residues were examined as described for the nucleotide data, with the exception that a Protopars weighting matrix was established for all amino-acid changes using MacClade²⁴. GenBank accession numbers U54472–U54495. Chernobyl and control localities were equivalent in sampling area. Wind patterns during the 10 days that the reactor emitted pollution produced an uneven distribution of radiation and other contaminants⁴. Our control site was selected because our instruments⁷ and radiation-distribution maps⁴ suggested low levels of radiation, and because the area contained a broad expanse of habitat suitable for voles. All individuals studied were collected in May and August 1994. Voucher specimens are deposited in the Museum of Texas Tech University.

populations and the Chernobyl populations. In the resulting trees, significantly more nucleotide substitutions were present in the Chernobyl samples than in the control samples (Fig. 1*a* and Table 1) (*M. rossiaemeridionalis* $\chi^2 = 13.8$, $P < 0.0003$; *M. arvalis* $\chi^2 = 20.2$; $P < 0.00001$). The inferred cytochrome *b* amino-acid sequence of each Chernobyl vole was unique, differing from the sequence of every other vole in the sample (Fig. 1*b*). This level of variation in amino-acid order is unprecedented among the populations of mammals studied so far^{8,9} and it is surprising that this degree of amino-acid evolution is tolerated in the Chernobyl population. In contrast, four of the five control sequences are identical in each species, a level of variation similar to that seen in cytochrome *b* in other rodents^{8,9}.

Estimating the amount of variation present in the Chernobyl population before 1986 as the amount of variation present in the control samples, and subtracting the control total from the total present in the Chernobyl sample, we estimate excess base-pair substitutions as 25(28 – 3) in *M. arvalis* and 17(19 – 2) in *M. rossiaemeridionalis*. Invoking the highly conservative assumption that all nine voles represent independent lineages and estimating the number of generations per year as two (estimated total of 144 generations), the nucleotide-substitution rate for the 1,143-bp gene for 42 mutations is calculated as 2.5×10^{-4} substitutions per nucleotide site per generation. Calculated separately, the rate for *M. arvalis* is 2.7×10^{-4} and for *M. rossiaemeridionalis* is 2.3×10^{-4} substitutions per nucleotide site per generation.

Although our estimates from two separate species at Chernobyl are similar, mutation rates of 10^{-4} are so exceptional (mutation rates for mitochondrial genes are generally 10^{-6} to 10^{-8} per

TABLE 1 Nucleotide substitutions arranged by codon position and effects of amino-acid substitution in two species of voles

Taxon and location	Codon position			Amino-acid effect	
	1st	2nd	3rd	Syn	Non
Chernobyl					
<i>M. rossiaemeridionalis</i>	6	5	8	9	10
<i>M. arvalis</i>	7	5	16	20	8
Total	13	10	24	29	18
Control					
<i>M. rossiaemeridionalis</i>	1	0	1	1	1
<i>M. arvalis</i>	2	0	1	2	1
Total	3	0	2	3	2

Syn, synonymous; Non, non-synonymous.

year)¹⁰⁻¹⁶ that independent confirmation is warranted. In particular, we wanted to investigate the possibility that these increased mutation/substitution rates resulted from immigration of heterogeneous colonists after the meltdown.

We therefore sequenced the cytochrome *b* gene from a Chernobyl-captured female *M. arvalis* which contained five embryos (Fig. 1; 'm' identifies the mother in the tree of *M. arvalis*). To control for polymerase error and to check for mosaicism, DNA was isolated from the mother's liver and heart, and two clones from each tissue were sequenced in both directions. No variation was found among these four clones. For each embryo, two clones were sequenced, and if any site differed from that of the mother (a potential mutation), a third independent amplification of that region from a new aliquot of DNA was sequenced to verify the presence of this base-pair substitution. No variation was detected among different clones from individual embryos. Two embryos had cytochrome *b* genes with nucleotide sequences identical to the mother. Three embryos were distinguished from their mother by one nucleotide change each; two individuals shared a third-position synonymous substitution, and one individual had a first-position non-synonymous substitution. Two independent (twinning, or a single mutation in the germ line before the two oocytes developed could account for the shared mutation) substitutions in 5,715 bp provide a substitution rate estimate of 3.5×10^{-4} , which corroborates the estimates from the populational comparisons above. Although our data are limited to a single female and her embryos, using the highest annual mutation rate normally accepted for the mitochondrial genome¹⁰⁻¹⁶ (1×10^{-6}), the probability of two mutations being observed by chance in 5,715 base pairs is 3.3×10^{-5} . Results from embryos suggest that the high substitution rate within the Chernobyl populations is ongoing, and contradict the hypothesis that the observed populational polymorphisms are the result of immigration. If this elevated mutation rate in cytochrome *b* extends to the entire mitochondrial genome (~17,000 bp), such a rate would translate into 3–5 substitutions per mitochondrial genome per generation. It is probable that such a high mutation rate does not extend to the nuclear genome because such a mutation rate across three billion base pairs would result in 600,000 mutations per gamete.

It must be understood that this increased substitution rate may reflect the presence of mutagens other than, or in addition to, radioactivity, or some synergistic effects of mutagens¹⁷. If these changes were caused solely by ambient levels of radiation, then the substitution rate should decline as the radioactivity decays. Although the level of ambient radiation has declined substantially since the accident¹⁸, our limited analyses of embryos suggest that the substitution rate is still vastly elevated over controls and may be similar to that which led to the current levels of genetic variation in the populations around the reactor. Unlike many of the radioisotopes released at Chernobyl, heavy metals and other mutagenic chemicals can persist indefinitely in the environment. In this and other respects, the environmental pollution resulting from the Chernobyl accident is different from that resulting from nuclear weapons. It does not appear that the biological consequences of the Chernobyl accident can be adequately predicted from results of previous laboratory studies^{19,20} or from the extensive investigations of the effects of Hiroshima and Nagasaki^{21,22}. □

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Essential role of the posterior morphogen nanos for germline development in *Drosophila*

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In many animal groups, factors required for germline formation are localized in germ plasm¹, a region of the egg cytoplasm. In *Drosophila* embryos, germ plasm is located in the posterior pole region and is inherited in pole cells, the germline progenitors. Transplantation experiments have demonstrated that germ plasm contains factors that can form germline²⁻⁴, and germ plasm also directs abdomen formation⁵. Genetic analysis has shown that a common mechanism directs the localization of the abdomen and germline-forming factors to the posterior pole⁶⁻¹². The critical factor for abdomen formation is the nanos (*nos*) protein (nanos)¹³⁻¹⁵. Here we show that *nos* is also essential for germline formation in *Drosophila*; pole cells lacking nanos activity fail to migrate into the gonads, and so do not become functional germ cells. In such pole cells, gene expression, which normally initiates within the gonad, begins prematurely during pole-cell migration. Premature activation of genes in germline precursors may mean that these cells fail to develop normally. A function for *nos* protein in *Drosophila* germline formation is compatible with observations of its association with germ plasm in other animals¹⁶⁻¹⁸.

Localized *nos* messenger RNA is translated *in situ* to form a gradient of *nos* protein with its highest concentration in germ plasm^{11,12,15,19}. The *nos* protein is only transiently present in the abdominal Anlagen, and becomes undetectable by the cellular blastoderm stage. In contrast, in germ plasm it is incorporated into pole cells and remains readily detectable throughout pole-cell migration until they reach the embryonic gonads¹⁵. These observations led us to speculate that *nos* protein may also be essential for germline formation. We investigated whether pole cells lacking nanos activity can develop into germ cells. Females homozygous for the maternal-effect *nos* mutation produce progeny that lack nanos activity¹³⁻¹⁵ and form pole cells^{13,14}. However, it is not clear whether these pole cells can condense in the embryonic gonads, and give rise to functional germ cells. This is because

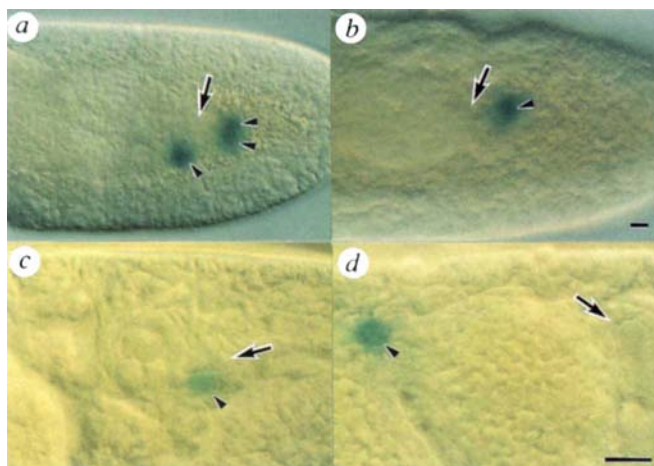


FIG. 1 The developmental fate of transplanted *nos* pole cells. In the text, we refer to embryos from *nos^{BN}/nos^{BN}* females as *nos* embryos, and to the pole cells formed by these embryos as *nos* pole cells, irrespective of zygotic genotype, because they lack *nanos* activity¹⁵. Pole cells formed in embryos from *nos^{BN}/+* females are designated as control pole cells. During normal embryogenesis, pole cells are formed at the posterior pole of

embryos lacking *nanos* activity have no abdominal mesodermal cells, which are required to assemble with pole cells to form gonads; one of the effects of *nos* mutation is the inability to form abdominal segments.

To determine whether *nos* pole cells can differentiate as germ line, we examined the developmental fate of pole cells transplanted from *nos* embryos into host embryos that can form normal abdomens. The transplanted *nos* pole cells migrated into the haemocoel, just as control pole cells did (Fig. 1a, b). However, none of the transplanted *nos* pole cells were incorporated in the gonads of the hosts (Table 1, Fig. 1d). In contrast, pole cells taken from the control embryos were found in the gonads, the midgut

cellularized embryos and migrate through the posterior midgut epithelium into the haemocoel, where they separate into two bilateral groups, and condense in the embryonic gonads (in the fifth abdominal segment) to differentiate as germ cells^{27,28}. To determine whether *nos* pole cells can differentiate as germ line, we examined the developmental fate of pole cells transplanted from *nos* embryos into *ovo^{D1}* host embryos. To identify the transplanted pole cells in the host embryos, a marker construct, *PLHΔ23*, which contains the *lacZ* gene under the control of a heat-shock promoter²⁴, was introduced into the donor embryos. The fate of the transplanted pole cells was followed by heat treatment, fixation and staining for β -gal at appropriate stages. Host embryos carrying *nos* pole cells (b and d) and control pole cells (a and c) were stained with X-gal. a, b, Embryos at stage 10 (stages as ref. 28). The *nos* and control pole cells (arrowheads) were located outside the midgut rudiments (arrows). c, d, Embryos at stage 15. The control pole cell was incorporated into the gonads (c), but none of the transplanted *nos* pole cells were incorporated in the host gonads. The *nos* pole cells were scattered in the midgut and hindgut lumen and in the haemocoel of the posterior half of the embryos (d). Arrowheads and arrows point to the transplanted pole cells and the gonads, respectively. Scale bars, 10 μ m.

METHODS. Pole-cell transplantation was as described in Table 1. After transplantation, hosts were kept at 25 °C until stage 10 or 15. The hosts were heat treated at 36 °C for 30 min, followed by incubation at 25 °C for 1 h, then were stained with X-gal as previously described²⁴. Transplanted pole cells were stained green with X-gal. Gonads were identified by morphological features: two dorsolateral distinctive cell clusters located in abdominal segment 5 (refs 27,28).

lumen and the haemocoel (Table 1, Fig. 1c). These results indicate that *nos* pole cells cannot penetrate the gonads.

To test whether *nos* pole cells contribute to egg production in adult females, we transplanted them into embryos carrying the dominant female sterile mutation *ovo^{D1}*. All female progeny are expected to be sterile unless they have received functional pole cells. None of the females that were transplanted with *nos* pole cells produced any progeny, whereas 16.4% of females that had received control pole cells were fertile and produced gametes derived from the transplanted pole cells (Table 2). Thus the autonomous deficiency of *nanos* activity in pole cells leads to their inability to penetrate the gonads and, consequently, results in their failure to become functional germ cells.

These results suggest that removal of *nanos* activity results in some change in gene regulation in pole cells, rendering them unable to penetrate the gonads. To understand this better, we examined the expression of three independent enhancer-trap markers, which show β -gal staining in pole cells incorporated in the gonads (see Fig. 2 for details). In control embryos, β -gal expression in pole cells became detectable at stage 13–14, when pole cells are incorporated in the gonads (Fig. 2a, c, e). The proportions of embryos with stained pole cells reached a maximum at stage 16, when maternal *nos* protein becomes undetectable in pole cells¹⁵. In contrast, in *nos* embryos these markers were prematurely expressed in pole cells during the course of their migration (Fig. 2a, d, f).

The above experiments show that the absence of *nanos* activity leads to the premature activation of marker genes normally expressed in pole cells within the gonads. In the pathway leading to abdominal segmentation, graded *nos* protein in the posterior half of embryos generates abdomen by repressing translation of maternal *hb* transcript distributed throughout the embryo^{20,23}. Similarly, the premature expression of the marker genes in *nos*

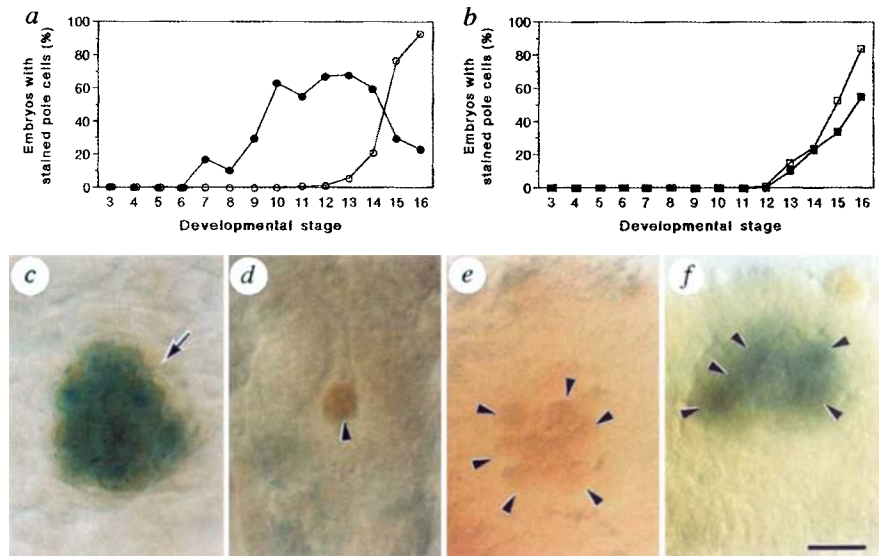
TABLE 1 The ability of transplanted pole cells to enter gonads

Genotype of females producing donor	Transplants	Surviving embryos	Embryos with labelled cells	Embryos with labelled cells in gonads (%)
<i>nos/TM3</i>	505	130	66	24 (36.4)
<i>nos/nos</i>	309	129	45	0
With <i>hb(Δ)</i>	186	76	45	19 (42.2)
Without <i>hb(Δ)</i>	162	75	44	16 (36.4)
Control		78	0	0

Genetically marked pole cells were transplanted into *ovo^{D1}* embryos. The full genotype of the *nos^{BN}* stock was *nos^{BN} e/TM3 e Sb Ser*. The *nos^{BN}* allele is caused by a P-element insertion into the promoter region of the *nos* gene¹⁵. *Nos^{BN}* produces a strong abdominal phenotype, but does not display the oogenesis defects associated with some *nos* alleles. Females homozygous for *nos^{BN}* produce embryos lacking *nos* RNA and *nos* protein¹⁵. Pole-cell donor embryos were derived from *nos^{BN}/nos^{BN}*, *nos^{BN}/TM3*, *w/w* females carrying the *hb(Δ)* construct²³, or *w/w* females without the *hb(Δ)* construct mated with *PLHΔ23* males. Because all embryonic cells with the *PLHΔ23* construct express β -gal after heat treatment²⁴, we can follow the fate of the transplanted pole cells during embryogenesis by heat treatment and staining for β -gal. To avoid contamination with somatic cells, we used late-syncytial blastoderm-stage embryos as donors. Pole-cell transplantation was performed basically as previously described²⁵. Pole cells (10–12) were transplanted from late-syncytial blastoderm donors to early-syncytial blastoderm hosts. The host embryos were derived from a cross between wild-type (Oregon-R) females and *ovo^{D1}* males. After transplantation, the hosts were kept at 25 °C until stage 15, were heat-treated at 36 °C for 30 min, followed by incubation at 25 °C for 1 h, then were stained for β -gal as described²⁴. The stained embryos were devitellinized manually, dehydrated in an ethanol series, and mounted in Eukitt. The embryos were observed under a compound microscope (DMRB, Leica) with Nomarski optics. The average number of labelled cells in a host that was ascertained to have stained pole cells was 2.6 ($n = 57$) when *nos* pole cells were transplanted, and was 3.1 ($n = 21$) when control pole cells were transplanted. The inability of *nos* pole cells to condense within the gonads does not result from selective death of these cells, as *nos* pole cells were as efficiently transplanted as control pole cells, and were present in comparable numbers in transplanted hosts. For controls, pole cells were not transplanted.

FIG. 2 Removal of *nos* activity results in the premature expression of late pole-cell marker, which normally appears in pole cells within the gonads. We examined the expression of an enhancer-trap marker, 198, which shows β -gal staining only in pole cells incorporated in gonads (Y. Obara and S.K., unpublished data). This marker was crossed to *nos^{BN}/nos^{BN}* females and its pattern of expression determined in the progeny. *a, b*, Stage-dependent expression of β -gal in pole cells in embryos produced from *nos^{BN}/nos^{BN}* (filled circles), *nos^{BN}/TM3* (open circles) females and w/w females with (filled squares) and without *hb(Δ)* (open squares) mated with the 198 enhancer-trap line. The frequency of embryos with stained pole cells is plotted against the stage. Embryos (10–157) of each genotype were examined at each developmental stage. In control embryos produced from *nos^{BN}/TM3* females, β -gal expression in pole cells was detected at stage 13–14, when pole cells were incorporated in the embryonic gonads, compared to stage 7–8, when pole cells begin moving into the embryo with the proctodeal invagination, in *nos* embryos. Similar results were obtained with two other independent enhancer-trap lines (data not shown). *c–f*, Embryos derived from *nos^{BN}/TM3* (*c* and *e*) and *nos^{BN}/nos^{BN}* females (*d* and *f*) mated with the 198 enhancer-trap line were double stained with X-gal (green) and an antibody against *vasa* protein, a marker protein specific to pole cells (orange). *c*, Pole cells incorporated in the gonad (arrow) of a stage-15 embryo were double stained. In the embryo, almost all pole cells in gonads were stained. *d*, A pole cell (arrowhead) in the hindgut lumen of a stage-15 embryo was not stained with X-gal. *e*, Pole cells (arrowheads) in the midgut rudiment of a stage-9 embryo were not stained with X-gal. *f*, Pole cells in the midgut rudiment of a stage-9 embryo were double stained (arrowheads). Scale bar, 10 μ m.

METHODS. Three enhancer-trap lines (198, 640 and 4351) were screened out from over 1,600 enhancer-trap lines carrying single P-lwB constructs



(Y. Obara and S.K., unpublished data). These enhancer-trap lines express β -gal in pole cells incorporated in gonads; 198 and 640 are homozygous viable and fertile, but 4351 is homozygous lethal. Embryos produced from *nos^{BN}/nos^{BN}*, *nos^{BN}/TM3* females and w/w females with and without *hb(Δ)* construct mated with the 198 enhancer-trap line were double stained with X-gal and an anti-*vasa* antibody as described previously²⁹. The dechorionated embryos were fixed in heptane saturated with 4% formaldehyde, then in heptane and 50% methanol. Fixed embryos were devitellinized with a tungsten needle and were processed for immunoperoxidase staining with an anti-*vasa* antibody. Immediately before peroxidase colour development, embryos were stained with X-gal, then peroxidase staining was resumed. The stained embryos were dissected and mounted in Eukitt.

pole cells can be explained by a failure of *nos* protein to suppress a regulatory pathway that is responsible for the expression of the late pole-cell markers. By analogy, this pole-cell-specific regulatory pathway could be mediated by a regulatory interaction between *nos* protein and *hb* RNA; however, the following observations demonstrate that this is not the case.

The ability of *nos* protein to regulate *hb* is mediated by discrete target sites, or *nos* response elements (NREs), in the 3' untranslated region of the *hb* transcript²³. Deletion of NREs from *hb* mRNA overrides *nos*-mediated repression, and embryos carrying this altered transcript *hb(Δ)* express *hb* protein throughout their entire length, including pole cells, and develop as embryos lacking abdominal segments²³. We examined whether premature expression of the late pole-cell markers is observed in the embryos expressing *hb(Δ)* transcript; no premature expression was discernible (Fig. 2*b*). During late embryogenesis, the pole cells expressed the markers without being enclosed by gonadal mesoderm. These observations show that *hb* protein could not direct the premature expression of the late pole-cell markers. Furthermore, pole cells derived from *hb(Δ)* embryos condensed in the gonad just as well as the normal pole cells (Table 1) if they were transplanted into a host that produced abdominal mesoderm.

Our results imply that *nos* protein in pole cells is not required for expression of the late pole-cell markers, but is essential to define the stage of expression. Another regulatory factor may be required to activate expression of the late pole-cell markers, with *nos* protein acting to repress expression. Although we cannot

TABLE 2 Contribution of transplanted pole cells to progeny production

Genotypes of females producing donor	Transplants	Surviving females	Fertile females producing progenies derived from donor pole cells (%)
<i>nos/TM3</i>	857	67	11 (16.4)
<i>nos/nos</i>	702	78	0
Control	—	109	0

Pole-cell transplantation was as described in Table 1. The *ovo^{D1}* females are sterile and do not produce eggs²⁶. The hosts were kept at 25 °C. The hatched larvae were transferred to a standard culture medium and allowed to develop to adult flies; they were mated and their fertility was examined. All female progeny are expected to be sterile, unless they have received functional pole cells. To confirm that the progeny of the fertile host females were derived from the transplanted pole cells, the host females were crossed to *e/e* males, and the genotype of their progeny determined. Because the genotype of the transplanted pole cells was *nos^{BN} e/TM3 e Sb Ser* or *nos^{BN} e/nos^{BN} e*, they produce the progeny homozygous for *e*. For controls, pole cells were not transplanted.

exclude the possibility that *nos* protein in pole cells directly represses the expression of the late pole-cell markers, we suggest that *nos* protein represses the production of the activator of marker expression, presumably by a translational control mechanism. Our results also show that ectopic overexpression of *hb* protein in pole cells does not result in premature expression of the late pole-cell markers, suggesting that maternal *hb* mRNA is not the only regulatory target of *nos* protein in pole cells. It is likely that regulatory factor(s) other than *hb* protein, which are responsible for the activation of late pole-cell markers, are stored in pole cells as mRNA(s) whose translation is repressed by *nos* protein. Once maternal *nos* protein is degraded in pole cells within the gonads, the mRNA(s) are translated to produce protein(s), which in turn activate late pole-cell enhancers.

It will be interesting to determine whether *nos* protein acts as part of an evolutionarily conserved mechanism of germ line

development, as *nos*-related mRNAs are associated with germ line in dipteran insects¹⁶ and the frog^{17,18}. We propose that localized nanos activity has a widespread role in germ-line development, in addition to its role in the establishment of embryonic asymmetry. □

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Defects in cardiac outflow tract formation and pro-B-lymphocyte expansion in mice lacking *Sox-4*

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A STRIKING example of the relationship between regulation of transcription and phenotype is the central role of the Y-chromosomal gene *Sry* in mammalian sex determination^{1,2}. *Sry* is the founding member of a large family of so-called *Sox* genes^{1,3}. During murine embryogenesis, the transcriptional activator *Sox-4* is expressed at several sites, but in adult mice expression is restricted to immature B and T lymphocytes⁴. Using targeted genedisruption, we have found that *Sox-4*^{-/-} embryos succumb to circulatory failure at day E14. This was a result of impaired development of the endocardial ridges (a specific site of *Sox-4* expression) into the semilunar valves and the outlet portion of the muscular ventricular septum. The observed range of septation defects is known as 'common arterial trunk' in man. We studied haemopoiesis in lethally irradiated mice reconstituted with *Sox-4*^{-/-} fetal liver cells and found that a specific block occurred in B-cell development at the pro-B cell stage. In line with this, the frequency and proliferative capacity of IL-7-responsive B cell progenitors in fetal liver were severely decreased *in vitro*.

In the heart at embryonic day E13, *Sox-4* is expressed exclusively in the endocardial cushions and ridges (Fig. 1). After disruption of the *Sox-4* gene (Fig. 2), no homozygous mutant offspring were born. At E13, *Sox-4*^{-/-} embryos were macroscopically indistinguishable from their littermates. They then rapidly

developed generalized oedema and died at E14. In moribund mutant embryos, heart rate increased while the blood was oscillating, suggesting a valvular insufficiency. Histological analysis of 12 mutant embryos consistently revealed dysplasia of the semilunar valves (Fig. 3). Furthermore, a large septation defect affected the entire outlet portion of the ventricles and, to a variable extent, the great arteries. This spectrum of cardiac development defects was indistinguishable from common arterial trunk type I (Fig. 3c–e) and type II (Fig. 3a, b) in man⁵.

The semilunar valves develop from the upper part of the endocardial ridges. Neural crest tissue is thought to be an important contributor to the proper development of the arterial pole of the heart, including the endocardial ridges⁶. The lower part of the endocardial ridges of the outflow tract is invaded by cardiomyocytes to form the outlet portion of the muscular interventricular septum⁷. To separate left and right circulation, the endocardial ridges fuse. Common arterial trunk type II will result, when the ridges remain unfused⁸. When additionally the aortopulmonary septum is not formed, common arterial trunk type I will result⁵.

In E13/14 *Sox-4* mutant embryos, the myocardium was not primarily affected by the mutation (Fig. 3f, g). The endocardial cushions were present and yielded normal atrioventricular valves (Fig. 3f). The endocardial ridges, however, never fused and failed to develop into proper semilunar valves. The absence of functional semilunar valves explains the oscillations of the blood observed in dying E14 embryos.

We conclude that the cardiac phenotype is the consequence of a primary defect in endocardial ridge development. This conclusion is based on two observations. (1) The ridges are specific (although not exclusive) sites of *Sox-4* expression, and (2) development of the semilunar valves from the ridges and fusion of these ridges is impaired. We suggest that the impaired development of the neural-crest-derived aortopulmonary septum is a consequence of the defect in the endocardial ridges, possibly due to the disturbance of the inductive processes between the ridges and the ingrowing neural crest-derived cells. Although several other gene disruptions interfere with cardiac septation^{8–13}, the features of the *Sox-4* mutation are unique in that they are confined to the arterial pole of the heart.

To investigate *Sox-4*^{-/-} haemopoiesis, fetal liver cells of E13 embryos (H-2^b) were injected into lethally irradiated MHC disparate recipients (H-2^{h/d}). In all mice, normal numbers of granulocytes and monocytes of donor origin were observed (not shown). After 8 weeks, lymphoid tissues were analysed. Donor-derived T lymphocytes (60–75%) were present, independent of the *Sox-4* genotype of the transplant (Fig. 4a, b). In contrast, CD43⁺B220⁺ pre-B cells were almost completely absent, whereas numbers of CD43⁺B220⁺ pro-B cells were reduced in *Sox-4*^{-/-}

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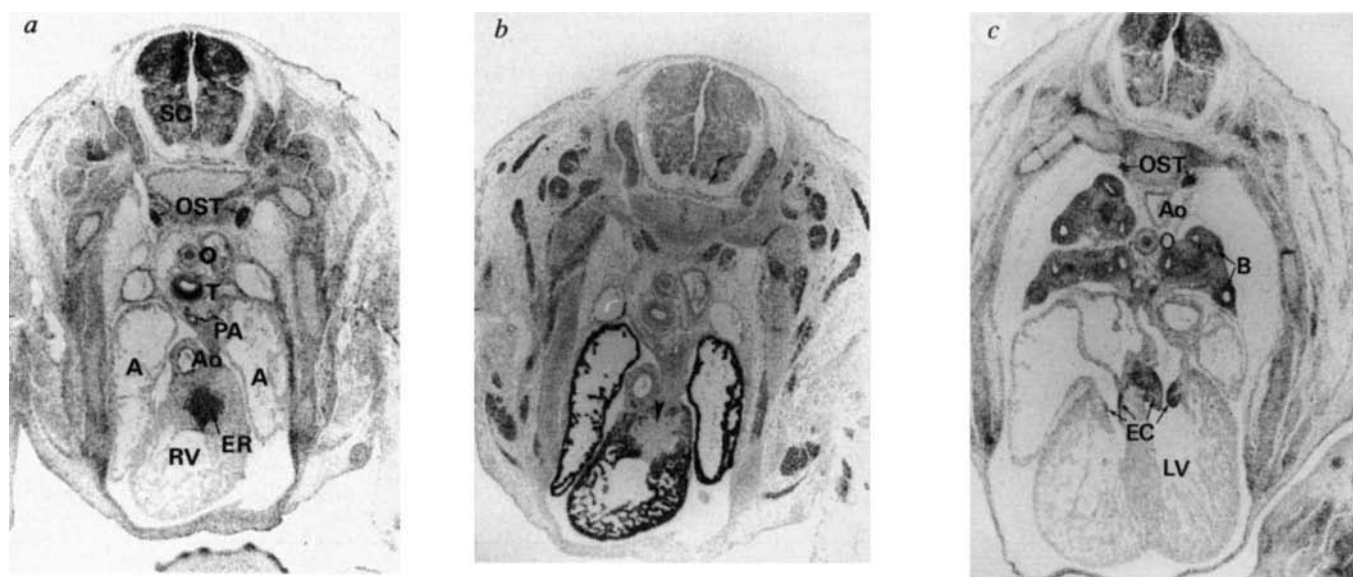


FIG. 1 Expression of Sox-4 in E13 embryos. Transverse sections of E13 embryos probed for Sox-4 (a, c) or calcium-dependent ATPase (b, d)²⁷. a, The Sox-4 gene is expressed in the endocardial ridges (ER) of the outflow tract, cartilage of the trachea (T), orthosympathic trunk (OST) and spinal cord (SC). Indicated are also oesophagus (O), aorta (Ao), pulmonary arteries (PA), atria (A) and right ventricle (RV). b, (Ca²⁺)ATPase expression in the myocardium of atria and ventricles in a consecutive section. Endocardial ridges are identified by the specific lack of (Ca²⁺)ATPase expression (arrowhead). c, Sox-4 expression in the (EC) endocardial cushions of the atrioventricular canal, and in the cartilage of the bronchi (B) of the lungs. Indicated are aorta (Ao), orthosympathic trunk (OST), oesophagus (O) and left ventricle (LV). d, (Ca²⁺)ATPase expression in a consecutive section to c. Endocardial cushions of the atrioventricular canal specifically lack (Ca²⁺)ATPase expression.

METHODS. *In situ* hybridization has been described²⁷. The RNA probe was transcribed from linearized Sox-4 cDNA in pBluescript SK using T3 RNA polymerase and ³⁵S-UTP (Stratagene). The probe (300 bp) contained unique untranslated sequences from the 5' end of the mRNA (EcoRI-SacI).

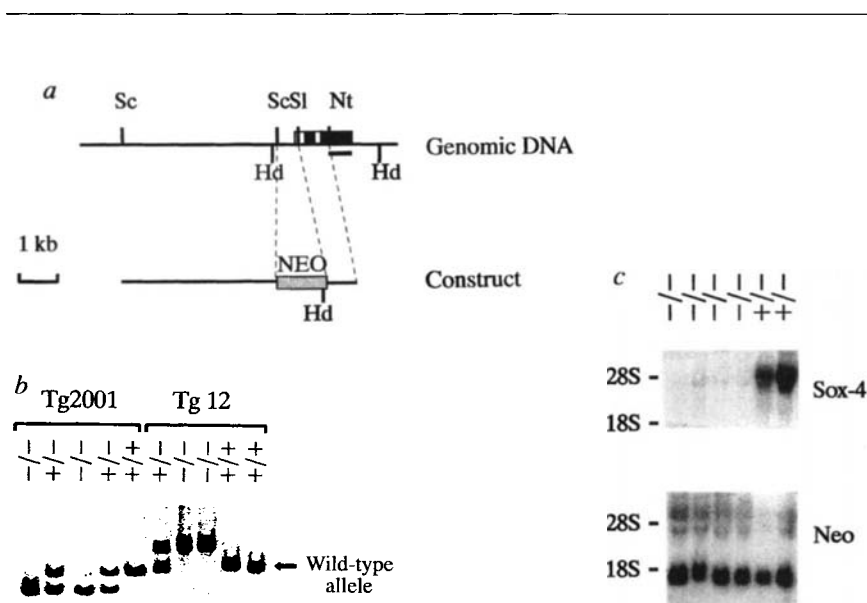


FIG. 2 Targeting of the Sox-4 gene. a, Structure of gene and construct used for homologous recombination. The coding sequence of the gene (top) comprises about 1,300 bp (boxed region). Positions of the HMG box (dark shading) and serine-rich stretch (lighter shading) are indicated. The construct lacks the initiator methionine. The PGK-neo' cassette was inserted in two orientations. A cDNA probe 3' of the NotI site (underline bar) was used as flanking probe to detect homologous recombination on DNA digested with HindIII. Restriction sites: Sc, SacI; Nt, NotI; Sl, SalI; Hd, HindIII. b, Identification of Sox-4^{-/-} mutant embryos by Southern blotting. Strain Tg12 contains the neo cassette in the sense orientation of the Sox-4 gene and in strain Tg2001 it is in the opposite orientation. Examples of wild-type, heterozygous and homozygous mutant embryos of both strains are shown. c, Northern blotting of RNA isolated from Sox-4^{+/+} and Sox-4^{-/-} embryos. RNA was isolated from the heads of E13 embryos, electrophoresed, blotted and hybridized with a 5' untranslated cDNA probe (Fig. 1). Lower panel shows hybridization of the same samples with a neo'-

coding probe as a control. Migration positions of RNA size markers (28S, 16S) are indicated.

METHODS. The Sox-4 gene was cloned from a mouse (129/Ola) genomic library²⁸. As the long arm of homology, a 4-kb SacI fragment was cloned in the SacI site of the pGEM-11 (Promega) vector. An XhoI-SalI digested PGK-neo cassette, containing the PGK promoter and the bovine growth hormone polyadenylation signal (from P. Soriano), was ligated in the SalI site of pGEM-11 in two orientations. Finally, an 800-bp SalI-NotI fragment from the Sox-4 coding sequence was ligated between the XhoI and NotI sites of pGEM-11 as the short arm of homology (oligonucleotides from Isogen, Amsterdam). After linearization with NotI, the construct was electroporated in E14 ES cells. G418 (300 µg ml⁻¹)-resistant clones were selected in the presence of buffalo rat liver cell conditioned medium. Germline transmission was obtained from two ES cell lines, with the PGK-neo cassette in opposite orientations, yielding identical phenotypes. Heterozygous mice were backcrossed to C57BL/6 for consecutive generations. Southern and northern blots were prepared according to standard procedures.

reconstituted bone marrow (Fig. 4c). During differentiation in the pro-B cell compartment, progenitors express increasing levels of heat-stable antigen (HSA)¹⁴. Pro-B cells are divided in fraction A (HSA^{low}), where D-J rearrangement starts to occur¹⁵, fraction B + C (HSA⁺), and fraction C' (HSA⁺⁺), in which most cells have rearranged the heavy chain gene^{14,15}. In the *Sox-4*^{-/-} bone marrow, fraction C' was virtually absent. Fraction B + C was decreased, whereas cells of fraction A were present in normal numbers. We also noted low numbers of CD43⁺B220⁺⁺ cells in *Sox-4*^{-/-} bone marrow (Fig. 4c), spleen and lymph nodes (Fig. 4a, b). These cells were donor-derived, surface-immunoglobulin-positive and represented mature B cells, indicating that the block in B-cell differentiation was not absolute.

In vitro, B-cell precursors in fetal liver that were responsive to interleukin-7 (IL-7) were reduced at least 10-fold in *Sox-4*^{-/-} embryos compared to littermates (Fig. 4d). Furthermore, *Sox-4*^{-/-} clones contained 10²–10³ cells after two weeks of culture,

whereas the control clones contained 10⁴–10⁵ cells. 29/30 heterozygous clones secreted immunoglobulin in response to lipopolysaccharide (LPS), indicating that B cells were present. None of seven mutant clones responded to LPS (Fig. 4e). Some *Sox-4*^{-/-} clones grew more rapidly after 6 weeks with IL-7 and became LPS-responsive (results not shown). Taken together *Sox-4*^{-/-} fetal livers not only contain less IL-7-responsive precursors, but these cells also expand much more slowly than control cells.

Where should the *Sox-4* gene be positioned in the molecular hierarchy controlling B-cell development? The hallmark of B-lineage development is the ordered rearrangement of the immunoglobulin genes and the assembly of functional receptor complexes on the cell surface. Mutations that interfere with surface expression of the immunoglobulin heavy chain^{15,16} result in an accumulation of cells in fraction B + C, and normal frequencies of IL-7 clonable precursors^{14,17,18}. In contrast, *Sox-4*^{-/-}

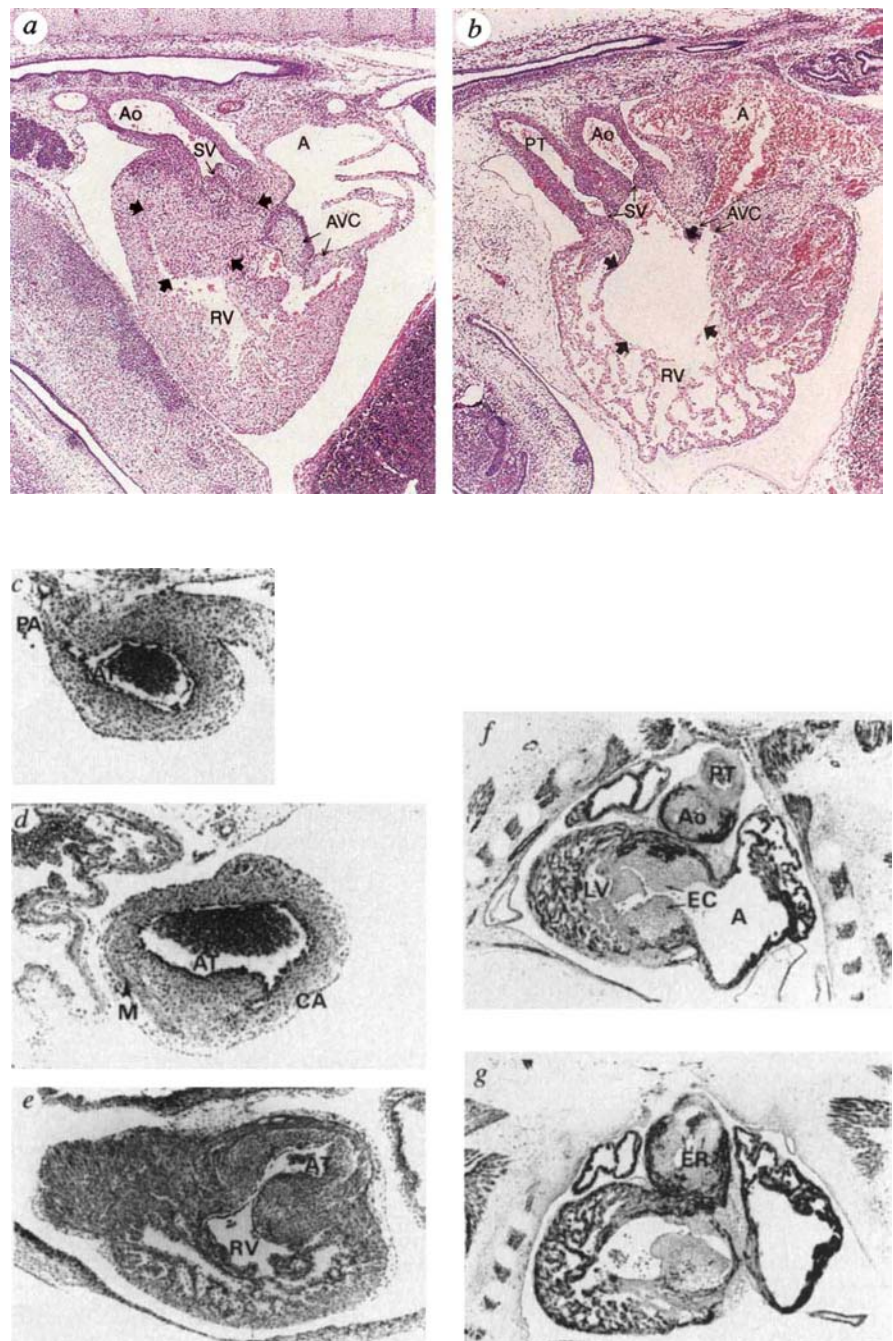


FIG. 3 Cardiac abnormalities in *Sox-4*^{-/-} embryos. *a, b*, Sagittal sections (haematoxylin-eosin (HE) staining) of normal (*a*) and mutant (*b*) E14 embryos show that in this *Sox-4*^{-/-} embryo the outlet portion of the muscular ventricular septum has failed to develop from the endocardial ridges (thick arrows). The semilunar valves are severely dysplastic. This particular heart represents an example of common arterial trunk type II. Aorta and pulmonary trunk are present, but both connect to the right ventricle. The atrioventricular valves (tricuspidalis) are normal. The thinning of the myocardium (sometimes present at E14) was never observed at E13 and therefore probably represented a secondary effect of the mutation. *c-e*, Transverse sections (haematoxylin-azophloxine (HA) staining) at different levels of a mutant heart (E14) representing a common arterial trunk type I. There is only one arterial trunk. It originates from the right ventricle (*e*), giving rise to both coronary arteries (*d*) as well as pulmonary arteries (*c*), and proceeds as aorta. *f, g*, Frontal sections of a mutant heart (E13) stained for smooth muscle α -actin, demonstrating that endocardial cushions (*f*) as well as the myocardium are normal (*f, g*) at E13. Although the endocardial ridges (*g*) will not develop into semilunar valves, the available mass of the ridges prevents regurgitation of the blood at this stage, explaining why circulatory failure occurs as late as E14. A, atrium; Ao, aorta; AT, common arterial trunk; EC, endocardial cushions of atrioventricular canal (AVC); CA, coronary artery; DA, ductus arteriosus; ER, endocardial ridges; LV, left ventricle; M, myocardium; PA, pulmonary artery; PT, pulmonary trunk; SV, semilunar valves; RV, right ventricle.

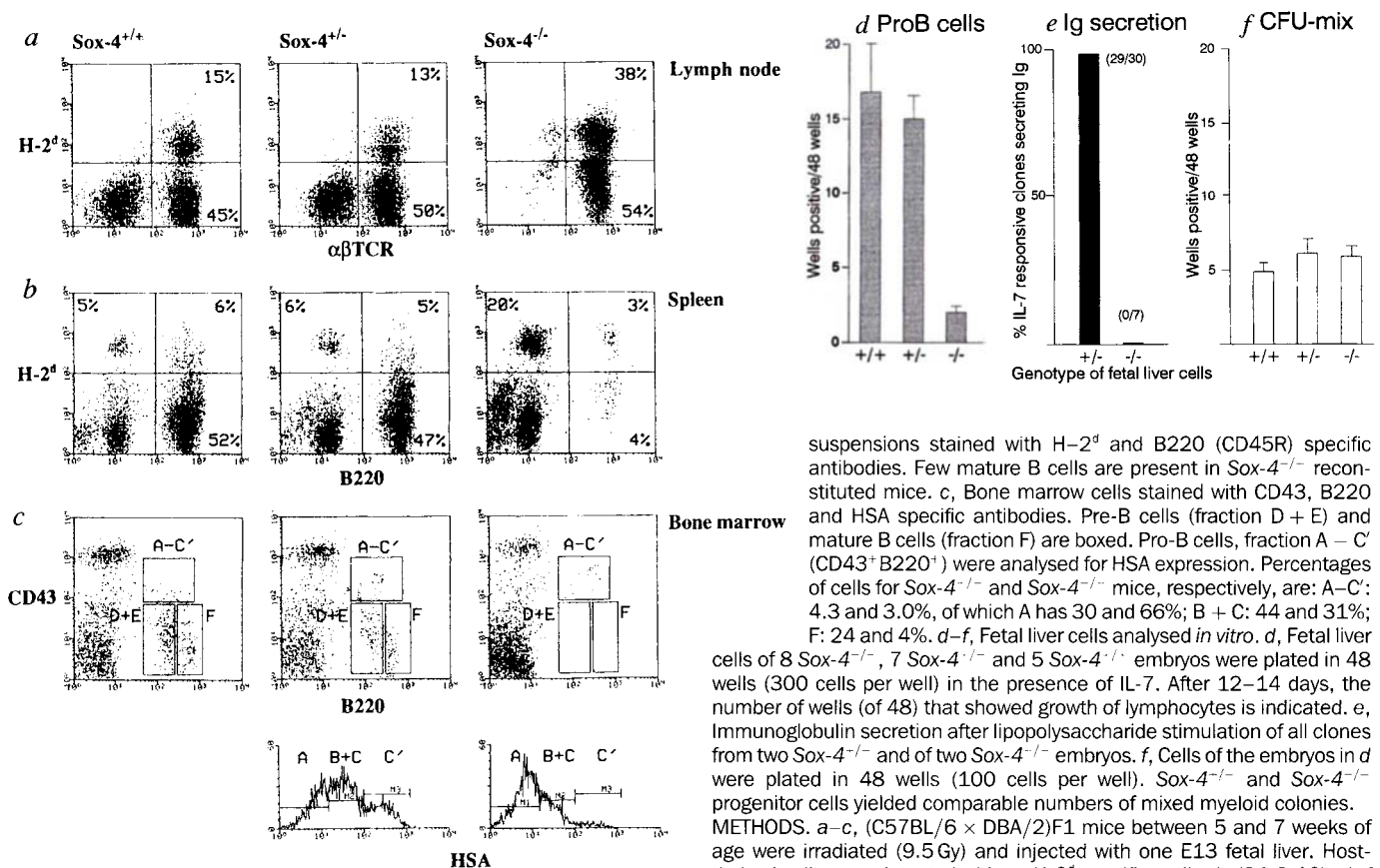


FIG. 4 B-cell development of *Sox-4*^{-/-} progenitor cells is impaired *in vivo* and *in vitro*. *a-c*, Lymphoid cells of bone marrow irradiation chimerae analysed by FACS eight weeks after reconstitution. *a*, Lymph node cell suspensions stained with H-2^d and αβ T-cell receptor specific antibodies. T cells are generated in nearly normal numbers from *Sox-4*^{-/-} progenitor cells. Note the virtual absence of non-T cells in the mutant. *b*, Spleen cell

bone marrow shows a decrease in fraction B + C (Fig. 3c), and the responsiveness of B-cell progenitors to IL-7 in *Sox-4*^{-/-} fetal liver is strongly diminished. The proliferative capacity of *Sox-4*^{-/-} pro-B cells is affected at a stage not yet dependent on surface expression of immunoglobulin heavy chain. This defect resembles the B-lineage defect in IL-7 receptor and IL-2R γ-chain mutant mice^{19,20} and suggests that the *Sox-4* gene product is a component of the pathway that controls pro-B-cell expansion.

Disruption of several other transcription factor genes has affected the B lineage. A tentative hierarchy of such factors can be proposed. Mutation of the *Ikaros* zinc-finger gene results in the

suspensions stained with H-2^d and B220 (CD45R) specific antibodies. Few mature B cells are present in *Sox-4*^{-/-} reconstituted mice. *c*, Bone marrow cells stained with CD43, B220 and HSA specific antibodies. Pre-B cells (fraction D + E) and mature B cells (fraction F) are boxed. Pro-B cells, fraction A - C' (CD43⁺B220⁺) were analysed for HSA expression. Percentages of cells for *Sox-4*^{-/-} and *Sox-4*^{+/-} mice, respectively, are: A-C': 4.3 and 3.0%, of which A has 30 and 66%; B + C: 44 and 31%; F: 24 and 4%. *d-f*, Fetal liver cells analysed *in vitro*. *d*, Fetal liver cells of 8 *Sox-4*^{-/-}, 7 *Sox-4*^{+/-} and 5 *Sox-4*^{+/+} embryos were plated in 48 wells (300 cells per well) in the presence of IL-7. After 12-14 days, the number of wells (of 48) that showed growth of lymphocytes is indicated. *e*, Immunoglobulin secretion after lipopolysaccharide stimulation of all clones from two *Sox-4*^{-/-} and two *Sox-4*^{+/-} embryos. *f*, Cells of the embryos in *d* were plated in 48 wells (100 cells per well). *Sox-4*^{-/-} and *Sox-4*^{+/-} progenitor cells yielded comparable numbers of mixed myeloid colonies. METHODS. *a-c*, (C57BL/6 × DBA/2)F1 mice between 5 and 7 weeks of age were irradiated (9.5 Gy) and injected with one E13 fetal liver. Host-derived cells were detected with an H-2^d specific antibody (34-2-12). *d-f* Primary cultures were prepared as described²⁹. For the detection of mixed myeloid colonies, 100 cells per well were plated in 96-well plates with MGF (Genetics Institute), IL-3 (from F. Melchers), IL-11 and EPO (1 U ml⁻¹). For the detection of clonable B-cell precursors, 300 cells per well were plated on irradiated (30 Gy) S17 stromal cells (from K. Dorshkind) with IL-7 (A. Rolink) and IL-11 (R. Hawley³⁰).

absence of all lymphoid lineages²¹. Absence of functional EBF or E2A results in a B-lineage block, apparently preceding that of the *Sox-4* mutation, before or in the pro-B cell stage, as mice with this mutation generate no^{22,23} or only very few²⁴ B220⁺ cells. Mutation of the *Pax-5* gene²⁵ has yielded a B-lineage block comparable to that of *Sox-4*.

We have previously reported that disruption of the T-lymphocyte-specific HMG-box gene *Tcf-1* specifically blocks thymocyte expansion at the transition of CD4⁺CD8⁺ cells to the CD4⁺CD8⁺ stage²⁶. *Sox-4* and *Tcf-1* might thus be controlling similar processes in B- and T-lineage development, respectively. □

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Activation of medial temporal structures during episodic memory retrieval

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MEDIAL temporal lobe structures have been implicated in human episodic memory. Patients with medial temporal lesions show memory deficits¹⁻³, and functional neuroimaging studies have revealed activation in this region during episodic encoding and retrieval when data are averaged over a sample of subjects⁴⁻⁷. The relevance of such observations for memory performance has remained unclear, however. Here we have used positron emission tomography (PET) to examine cerebral blood flow related to verbal episodic retrieval. We observed strong positive correlations between retrieval and blood flow in left medial temporal structures in individual normal human subjects. In addition, multivariate analysis showed that regions in the left medial temporal lobe were dominant components of a pattern of brain regions that distinguished a high-retrieval condition from conditions of lower retrieval. These results suggest that medial temporal activity is related to retrieval success rather than retrieval attempt, possibly by reflecting reactivation of stored patterns.

The experiment included four conditions in which regional cerebral blood flow (rCBF) was estimated from PET counts⁸: visual recognition of words following meaning based encoding; visual recognition of words following perceptual encoding; visual recognition of non-studied words; and a baseline word-reading condition (Fig. 1). The pattern of rCBF during recognition of items following meaning-based encoding was cross-correlated with the proportion of correctly recognized items across individual subjects. This analysis specifically took advantage of individual variability in behavioural performance and pixel-by-pixel blood flow rate, which cannot be done in pairwise subtraction analyses of averaged images⁹.

A significant correlation between individual recognition performance and brain activity was observed in a region in the left anterior medial temporal lobe (Fig. 2). This was true for the data from both scans ($r_{\text{scan1}} = 0.80$; $r_{\text{scan2}} = 0.83$), suggesting that the correlation is reliable within this sample. Further support for the reliability of the relation was shown by a positive correlation between activity in this region and recognition performance following perceptual encoding ($r = 0.63$, $P < 0.05$). The strongest correlations between performance and rCBF in the perceptual condition were observed in left cerebellum and bilateral fusiform gyrus. The correlation between activity in the medial temporal region and the false-alarm rate in the non-studied condition was weakly negative ($r = -0.22$), suggesting that the relation between recognition performance for studied words and brain activity in this region reflects successful retrieval rather than a tendency to respond positively.

The outcome of the correlational analyses suggests a relation between verbal episodic retrieval and activity in the left medial temporal lobe. Subjects who correctly recognized many words tended to have high levels of activity in the anterior part of left parahippocampal gyrus. This demonstration of a brain-behaviour relation on the level of individual subjects adds to similar demonstrations in other domains^{10,11}, and is consistent with clinico-

0	Study task 1: Perceptual encoding of words (Set A1 and A2)
2	Study task 2: Meaning-based encoding of words (Set B1 and B2)
12	Scan 1: Reading of non-studied words (Set C1)
24	Scan 2: Recognition test of words from study task 1 (Set A1)
36	Scan 3: Recognition test of words from study task 2 (Set B1)
48	Scan 4: Recognition test of non-studied words (Set D1)
60	Scan 5: Recognition test of non-studied words (Set D2)
72	Scan 6: Recognition test of words from study task 2 (Set B2)
84	Scan 7: Recognition test of words from study task 1 (Set A2)
96	Scan 8: Reading of non-studied words (Set C2)

FIG. 1 The experimental procedure for one subject. At the beginning of the session, subjects were auditorily presented with two lists of 80 words each at the rate of one word every 1.5 s. Half of the words were read by a female voice, half by a male voice. In the perceptual-encoding task, subjects decided whether the words were read by a female or male voice. In the meaning-based encoding task they decided whether the words referred to living or non-living things. In the first and last PET scan, subjects silently read visually presented words. They arbitrarily pressed one of two computer mouse buttons after reading each word. In scans 2-7, they made yes/no recognition judgements as to whether visually presented words had been part of any of the study lists. They responded by pressing one of two computer mouse buttons. Meaning-based encoding led to higher levels of recognition ($M = 58\%$, $s.d. = 0.19$) than perceptual encoding ($M = 30\%$, $s.d. = 0.18$). The false-alarm rate in the non-studied condition was substantial ($M = 17\%$, $s.d. = 0.12$), which made it possible to test for the effect of false positive responses. The order of the recognition scans was counterbalanced across subjects (11 right-handed young volunteers). In all scans, the words were presented every 3 s in the centre of a computer screen, and they remained on the screen for 2 s. PET scans were obtained with a GEMS-Scanditronix PC 2048-15B head scanner using bolus injections of 30 mCi $H_2^{15}O$ and 60 s data acquisition interval. The study was approved by the Human Subjects Use Committee of Baycrest Centre; written informed consent was obtained from the subjects.

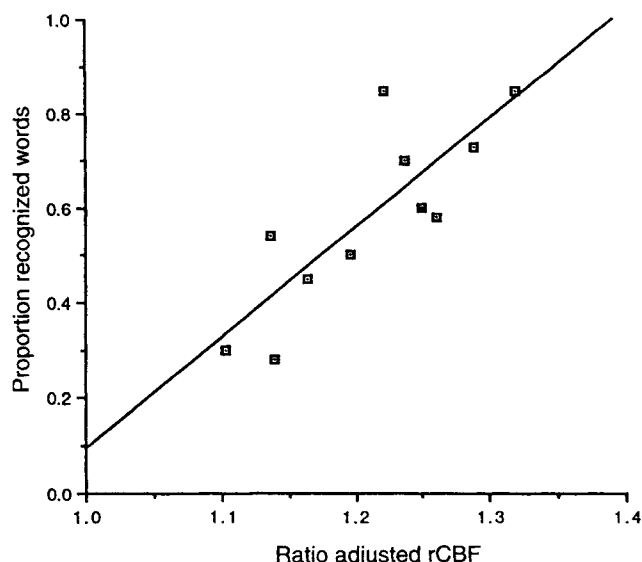


FIG. 2 Correlation between episodic-memory retrieval and standardized rCBF in left medial temporal lobe. Pearson's product moment correlations were computed between each subject's recognition performance in the meaning-based condition and individual patterns of rCBF. A strong correlation was found at a voxel in the left anterior medial temporal lobe (Talairach & Tournoux x, y, z coordinates²³, $-24, 2, -16$). The correlation for the proportion of correctly recognized words averaged across the two scans is shown [$r(9) = 0.82$, $P < 0.01$]. Other areas showing strong correlations ($r > 0.70$) included cingulate cortex ($-2, -16, 32$) and bilateral inferior prefrontal cortex ($-34, 30, -12$; $30, 36, -12$). Images were transformed to a common geometrical space using SPM-94, (ref. 24) and smoothed with a 10-mm isotropic gaussian filter. All covariance analyses were performed using PRO-MATLAB (version 4.2).

anatomical correlation studies in brain-damaged patients showing that left-sided medial temporal lesions produce verbal memory deficits¹². It also extends a previous finding of a relation in a study that looked at the correlation between group-averaged rCBF in the hippocampal formation and group-averaged free-recall performance¹³. The design of that study made it impossible to tell whether the relation reflected encoding processes, retrieval processes, or both.

The relation between activity in the left medial temporal lobe and episodic retrieval leads to the expectation that recognition following meaning-based encoding, which resulted in the highest memory performance, should be associated with higher activity in the left medial temporal lobe than the other experimental conditions. To test the expectation, we conducted a partial least-squares analysis¹⁴ of the relation between the four experimental conditions and the functional images from each subject. The analysis yielded

a spatial pattern of brain regions that distinguished between recognition following meaning-based encoding and the other three conditions. The dominant components, (those with the strongest positive weight) in this spatial pattern were located in the left and right medial temporal lobe (Fig. 3). This finding confirms the expectation of increased left medial temporal activity with increased retrieval, and is consistent with the results of a recent PET study comparing a high-recall and a low-recall condition⁶. By involving a shift in study-test modality, we have shown that medial temporal activity is associated with verbal, and not only visual¹⁵, retrieval. By identifying patterns of brain regions that discriminated between conditions, the analysis also points to the potential importance for successful retrieval of other brain structures, such as the temporal pole¹⁶, that together form a distributed network.

The above analyses converge on the notion that left medial

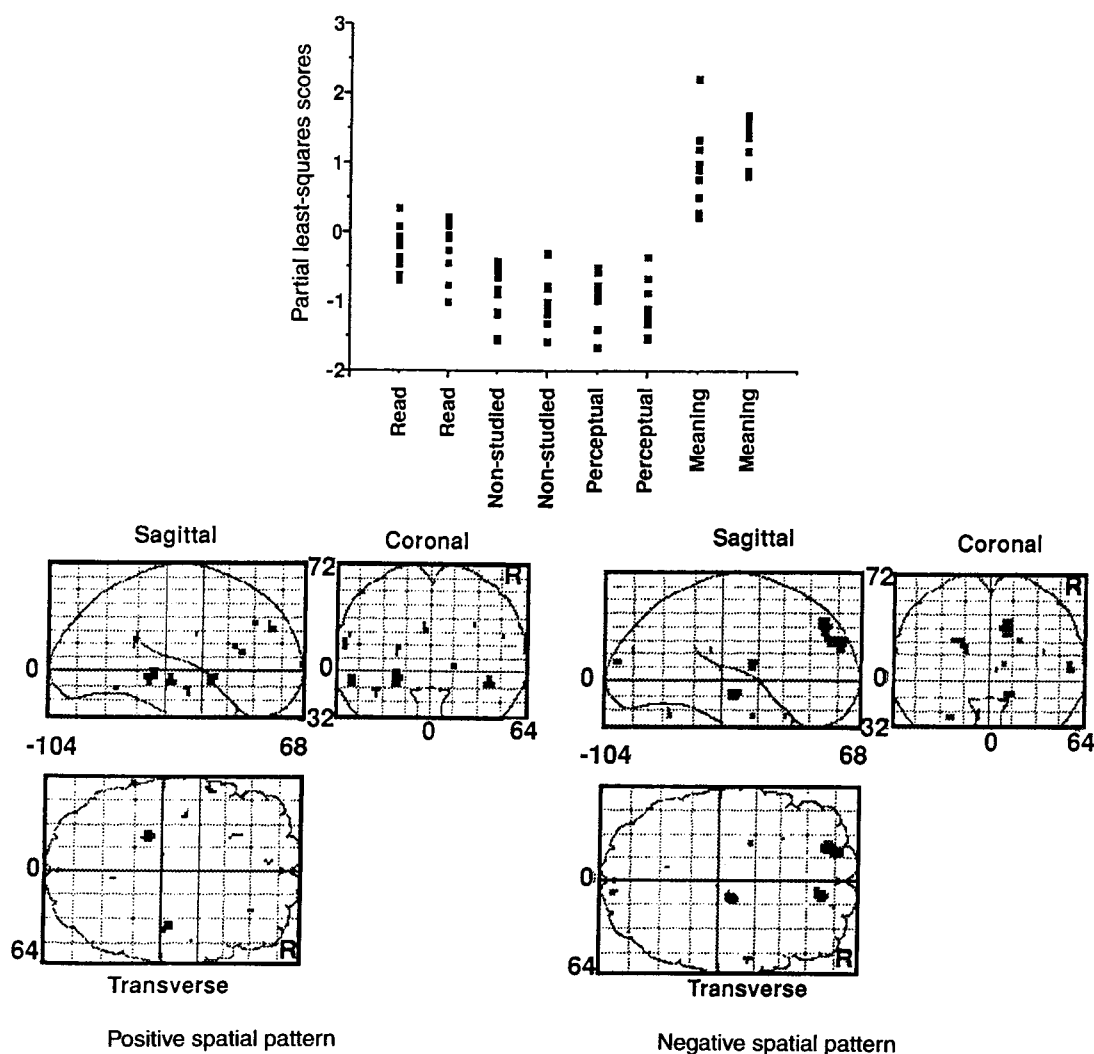


FIG. 3 Results of partial least squares analysis¹⁴. Top, for each subject in each scan, the scores on the spatial pattern that distinguished between recognition following meaning-based encoding and the other conditions. Permutation tests on these scores confirmed that the difference was significant ($R^2 = 0.72$, $P = 0.001$). Bottom, projections of voxels showing the highest weights for this pattern represented in three orthogonal dimensions on a transparent brain outline. Voxels contributing $> 20\%$ of their variance are shown. Positive weights were observed in the vicinity of left and right hippocampi (x, y, z coordinates²³, $-24, -36, -8$; $-36, -10, -16$; $38, -22, -12$), left temporal polar ($-54, 6, -8$), left caudate/insular ($-24, 26, 8$; $-22, 18, 12$), left superior temporal ($-58, -48, 12$), and right dorsal prefrontal ($28, 34, 28$) cortices. Negative weights were observed in bilateral dorsomedial prefrontal cortex ($-18, 52, 20$; $10, 42, 32$) and midbrain ($12, -20, -12$). The partial least squares analysis operates

through a singular value decomposition of a cross-correlation matrix between functional images for all subjects in all conditions and contrast vectors coding for the experimental design. The outcome is sets of mutually independent spatial patterns depicting brain regions that show the strongest relation to (that is, are most covariant with) the contrasts. Subjects scores on these patterns are computed by multiplying an individual's image within a condition by the voxel weights for a spatial pattern and summing the cross-products so there is a single score for each subject in each scan condition. The differences of these scores across conditions were tested by a multiple regression approach to the analysis of variance, and significance was assigned using permutation tests²⁵. The increased sensitivity of partial least-squares over univariate analyses arises because it deals with the entire image simultaneously.

temporal lobe activity is related to verbal episodic-memory retrieval. To test the generalizability of this outcome, we analysed the relation between rCBF and recognition memory performance using data from another sample of subjects. A significant correlation was found between individual memory performance and activity in a region in the left medial temporal lobe (Fig. 4). This finding provides further support that high activity in the left medial temporal lobe is related to successful episodic retrieval of verbal information. The spatial extent of this region of significant correlation overlapped with that identified by the partial least-squares analysis, but not the behavioural correlation, in the first data set. This lack of overlap may be related to either the less severe smoothing filtering we used for our data, or to the fact that the first study used auditory presentation¹⁷.

In the context of retrieval success, medial temporal lobe structures have been assumed to be involved in reactivating stored representations¹⁸. Our results have implications for this view by suggesting that it is not only the attempt of reactivation that leads to increased activity, but also successful reactivation. Reactivation of regions involved in storage may feed back to the medial temporal area through reciprocal connections¹⁹, leading to increased activity. In turn, this increased activity may underlie recollective experience or confidence (D.L. Schacter *et al.*, manuscript submitted). This hypothesis is consistent with previous demonstrations that damage to the medial temporal region primarily impairs memory performance on tests requiring conscious recollection of the study episode²⁰, and also with findings that confidence in the accuracy of retrieval varies as a function of level of performance²¹. Our results are also consistent with the view that events are initially stored in the hippocampal system²², in

this context the observed relation may reflect reinstatement of stored patterns. □

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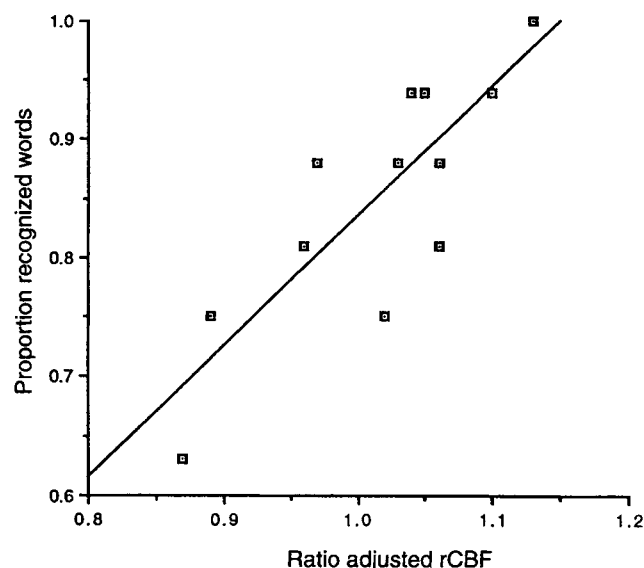


FIG. 4 Correlation between episodic-memory retrieval and rCBF near left hippocampus. Right-handed volunteers (12) intentionally studied 30 visually presented nouns. About 8 min later, subjects were PET scanned (bolus injection of 40 mCi of $H_2^{15}O$) while they performed yes/no recognition judgements for visually presented test words. Both at study and test the words were presented every 4 s and remained on the screen for 3 s. The subjects responded by pressing one of two computer mouse buttons with their right hand. The test list included 20 old words and 10 new words. Only old words were presented during the scan window, and subjects accurately identified most of them as old ($M = 85\%$, $s.d. = 0.10$). Pearson's product moment correlations were computed between each subject's recognition performance and individual patterns of rCBF. A strong positive correlation was found at a voxel near left hippocampus (x, y, z coordinates²³, $-24, -24, -12$, $r(10) = 0.82$, $P < 0.01$). The scan was part of a larger study, which will be reported elsewhere.

Prevention and reversal of renal allograft rejection by antibody against CD45RB

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REJECTION continues to be the single largest impediment to successful organ transplantation¹. Antilymphocyte globulin, which contains antibodies that react with the leukocyte common antigen known as CD45 (refs 2–6), has proved to be one of the most effective agents for preventing rejection. We have shown earlier that a monoclonal antibody directed against the RB isoform of CD45 substantially inhibits the alloreactivity of human CD4⁺ lymphocytes *in vitro*⁷. Here we investigate whether CD45RB could be an appropriate target for preventing renal allograft rejection in mice. Mice treated with two injections of a monoclonal antibody (MB23G2) (ref. 8) raised against CD45RB protein all survived and had normal renal function. Furthermore, this antibody reversed acute rejection when therapy was delayed until day 4, and the mice survived for their natural lifespan. The immunosuppression achieved may find application in the prevention and treatment of transplant rejection in man.

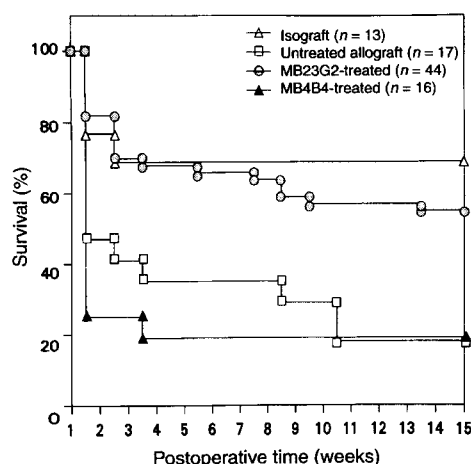


FIG. 1 CD45RB mAb MB23G2 induces prolonged allograft survival. There was no significant difference between the MB23G2-treated and isograft groups (log-rank test). There was a significant difference between these two groups, and the untreated and MB4B4-treated allograft recipients ($P < 0.002$). There was no difference between the MB4B4 and untreated allograft recipients.

Fully allogeneic renal allografts were performed with the use of C57BL/6 (H-2b) mice as donor and BALB/c (H-2d) mice as recipient. A two-stage procedure was undertaken in which the recipient's left native kidney was left in place and the right native kidney was removed at the time of transplantation⁹. The left native kidney was then removed on the seventh day after the operation. The animals were observed daily until they died. There were four groups of animals (Fig. 1): 13 that received isografts; 17 that received allografts with no immunosuppression (vehicle control); 44 that received allografts and were given two doses (1 mg kg^{-1} ; $30 \mu\text{g}$) of rat anti-mouse CD45RB monoclonal antibody MB23G2 (ref. 8) (ATCC, American-Type Culture Collection, Rockville, Maryland, USA) intravenously on days 0 and 1; and 16 that received allografts but were treated with two doses (1 mg kg^{-1} ; $30 \mu\text{g}$) of rat anti-mouse CD45RB monoclonal antibody MB4B4 (ref. 8) (ATCC) intravenously on days 0 and 1. No further immunosuppressive treatment was given.

The MB23G2-treated animals had prolonged survival compared with the untreated group ($P < 0.002$) (Fig. 1) and was comparable to that of the isograft group. Remarkably, the CD45RB antibody MB4B4 was no better than vehicle alone at preventing rejection. Both antibodies are IgG2a, but whereas MB23G2 recognizes a neuraminidase-sensitive epitope, MB4B4 binds to a neuraminidase-insensitive epitope⁸. Table 1 shows the serum creatinine levels in animals from each group on post-operative days (POD) 10 and 100. Creatinine levels in untreated and MB4B4-treated groups were significantly higher than those in mice with isografts and MB23G2-treated allografts on POD 10 ($P < 0.01$). There were no differences between the isografted and MB23G2-treated groups, but the untreated and MB4B4-treated animals died from uraemia. Other groups of animals were given $30 \mu\text{g}$ MB23G2 or MB4B4 intravenously

(i.v.) daily for two days and then $100 \mu\text{g}$ intraperitoneally (i.p.) for 9 more days. No difference in efficacy was noted when compared with just two doses of $30 \mu\text{g}$ given i.v. on days 0 and 1. Immunoperoxidase studies on the renal allografts (Table 1) showed that significantly fewer CD3⁺ and CD8⁺ T cells infiltrated MB23G2-treated kidneys at 7 days.

To assess the possibility of antigen-specific tolerance, we performed skin transplants on 13 animals which had maintained their kidney transplant 100 days beyond receiving two doses of MB23G2 antibody at the time of renal allografting. Each animal received full-thickness skin allografts from C57BL/6 mouse (isogenic with the donor of the renal allograft) and a control skin transplant: nine received a BALB/c isograft and four a CBA allograft (third-party donor). No further immunosuppressive treatment was given. Of the 13 animals with kidney-specific tolerance, a subset of four showed donor-antigen-specific tolerance because they did not reject the C57BL/6 skin. All four animals rejected the third-party CBA skin, whereas all nine BALB/c isografts survived. No renal allograft rejection was stimulated by the skin transplants.

To determine whether MB23G2 antibody could reverse acute rejection, seven allografts were made but no immunotherapy was administered until day 4. Untreated allografted kidneys were rejecting at this time. Treated animals received MB23G2 (1.5 mg kg^{-1} ($50 \mu\text{g}$) i.v.) daily on days 4, 5 and 6 but then no further therapy. Three animals died of ureteric complications in the MB23G2-treated group—graft histology showed that there had been no rejection at the time of death on days 8, 9 and 25. All animals had their rejection reversed and the remaining four survived > 60 days with normal levels of serum creatinine.

We assessed the pharmacological effects on the peripheral blood of mice treated with MB23G2 ($30 \mu\text{g}$ given i.v. on two consecutive days) by using multiparameter FACS analysis¹⁰. As

TABLE 1 Serum creatinine levels in allografted mice and cellular infiltration of renal transplants

	Isograft	Untreated allograft	MB4B4-treated allograft	MB23G2-treated allograft
Creatinine ($\mu\text{mol l}^{-1} \pm \text{s.e.m.}$) postoperative day 10	34 ± 5	144 ± 17	156 ± 26	$40 \pm 4^*$
Creatinine ($\mu\text{mol l}^{-1} \pm \text{s.e.m.}$) postoperative day 100	40 ± 6	ND	ND	54 ± 6
CD3 (KT3) infiltrates (median per HPF)	ND	33	38	22*
CD4 (GK1.5) infiltrates (median per HPF)	ND	10	11	9
CD8 (3.155) infiltrates (median per HPF)	ND	27	26	13*
CD45RB (MB23G2) infiltrates (median per HPF)	ND	12	10	9

Immunoperoxidase microscopic studies were performed on renal transplants at 7 days. Sections were stained with rat anti-mouse mAb reactive with mouse CD3, CD4, CD8, CD45RB and Ia. Slides were evaluated in a masked manner with respect to aggregates and diffuse infiltrates as described¹⁴. The numbers of cells in the diffuse infiltrates were counted in ten ($\times 400$) high-power fields (HPF) in each section of 5 mice per group. Differences between groups became evident after separately counting cells in aggregates and diffuse infiltrates. Aggregates contained high numbers of CD4⁺ and CD8⁺ all three groups. Numbers of CD3⁺ cells and CD8⁺ cells were statistically different between MB23G2-treated and untreated or MB4B4-treated groups in the diffuse infiltrates. Staining for Ia was clearly less in MB23G2-treated allografts, but owing to positive staining of other interstitial cell types, the numbers of lymphocytes could not be reliably quantified (data not shown). The numbers of CD4⁺ cells and CD45RB⁺ cells were approximately equivalent in all three groups. Remarkably, few of the infiltrating cells were CD45RB⁺, a finding that is particularly notable considering that the CD45RB mAb MB23G2 could reverse acute rejection.

ND, not determined. Creatinine values were not determined because most of the mice in these groups died before POD 100. Cellular infiltration was not determined in isografts because there were no infiltrating mononuclear cells.

* $P < 0.05$: statistically significant differences between MB23G2-treated and untreated and MB4B4-treated groups.

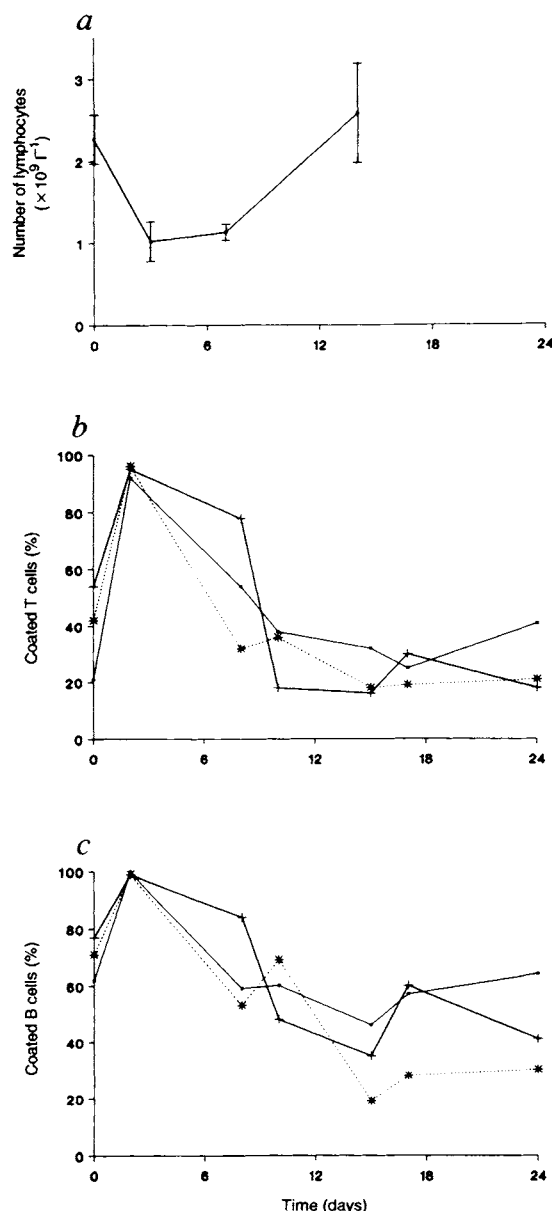


FIG. 2 MB23G2 depletes peripheral blood lymphocytes. **a**, Four groups of mice were treated with 30 μ g MB23G2 mAb intravenously on 2 consecutive days. One group of untreated animals was killed before therapy ($n = 3$) and the other three groups were killed after therapy on day 3 ($n = 6$), day 7 ($n = 3$) or day 14 ($n = 3$) and white blood cell and differential counts were analysed. The mean ($\pm$ s.e.m.) absolute lymphocyte count was then calculated. The difference between day 0 and day 14 is not significant. The difference between days 0/14 and days 3/7 is significant ($P = 0.05$ by Wilcoxon rank sum test). **b** and **c**, Three different mice (asterisks, crosses or circles) were treated with 30 μ g MB23G2 mAb intravenously on 2 consecutive days. Blood (100 μ l) drawn from a tail vein was collected in a heparinized tube on the indicated days. The red cells were lysed with ammonium chloride and the cells prepared for multiparameter FACS analysis¹⁰. Goat anti-rat fluorescein isothiocyanate was used to detect bound MB23G2. Anti-CD3-phycoerythrin was used to identify T cells (**b**) and goat anti-mouse phycoerythrin was used to detect B cells (**c**). Per cent coated cells represents the cells that were doubly stained with anti-rat Ig and the T- or B-cell-specific antibody. MB23G2 bound to almost all the remaining T and B lymphocytes that were not depleted. On day 2, FACS analysis revealed that the CD45RB antigen was saturated by the administered CD45RB antibody. FACS analysis revealed no MB23G2 antibody in the plasma by day 8 (ref. 10). FACS analysis of the spleen showed that the administered therapy penetrated the lymphoid tissue 24 h after the second dose of MB23G2. A FACS inhibition assay¹⁰ failed to reveal sensitization of any of the mice to the MB23G2 mAb two weeks after therapy.

shown in Fig. 2, MB23G2 caused a significant drop in the number of circulating lymphocytes, which returned to normal one week after stopping antibody treatment.

As CD45 is a protein-tyrosine phosphatase, we tested the idea that induction of tolerance by MB23G2 could be related to an alteration in tyrosine phosphorylation of T-cell substrates that is necessary for signal transduction. In the presence of the therapeutically effective MB23G2 antibody, tyrosine phosphorylation was increased in a substrate of relative molecular mass (M_r) 145K (Fig. 3), but MB4B4, which is ineffective, did not alter tyrosine phosphorylation of this substrate. Immunoglobulin was then stripped from the PVDF membranes and reprobed with monoclonal antibodies raised against phospholipase $C\gamma_1$ (PLC- γ_1), which proved that MB23G2 augmented tyrosine phosphorylation of this substrate. Increased tyrosine phosphorylation of PLC- γ_1 has been reported¹¹ to be a property of anergic T cells. We next assessed gene expression in the renal transplants by northern blot analysis¹² and found that MB23G2-treated animals had reduced levels of steady-state messenger RNA transcripts for γ -interferon, tumour necrosis factor- α and intercellular adhesion molecule-1 on day 28 when compared with untreated allografts on day 7.

We have identified a new type of immunotherapy for allograft rejection. It is perhaps not surprising that CD45RB plays a role in allograft immunity. CD45 represents 10% of total T-cell-membrane carbohydrate and a lack of CD45 renders the T cell incapable of responding to specific antigen¹³. Furthermore, CD45 is the principal reactive component in antilymphocyte globulin^{2,4}. During investigations of CD45 as a target for *in vivo* immunosuppressive therapy⁴, it was discovered that a purified antiserum against CD45 was not as effective as polyclonal antilymphocyte globulin in a rat allograft model, although graft survival was prolonged⁴. Furthermore, a monoclonal antibody

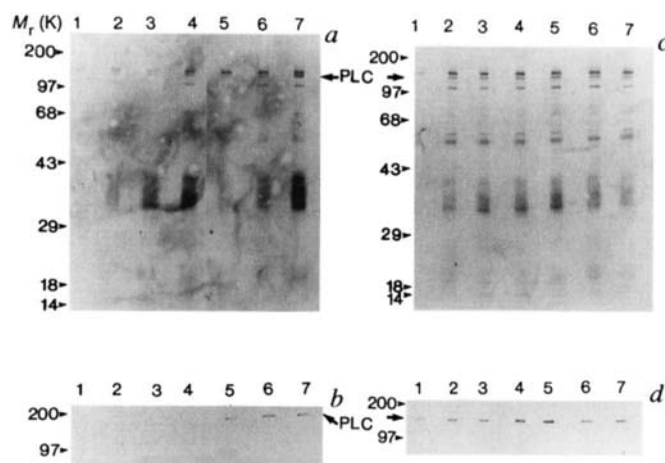


FIG. 3 MB23G2 mAb (against CD45RB) augments tyrosine phosphorylation of phospholipase $C\gamma_1$. The A1.1 T cell hybridoma was stimulated with anti-CD3 ϵ mAb 2C11 for 5 min at 37 $^{\circ}$ C and 1 μ g ml⁻¹ (lanes 2 and 5 in **a**–**d**), 2 μ g ml⁻¹ (lanes 3 and 6 in **a**–**d**), or 5 μ g ml⁻¹ (lanes 4 and 7 in **a**–**d**), or they were unstimulated (lane 1, in **a**–**d**). Cells were incubated with 50 μ g ml⁻¹ MB23G2 (lanes 5–7 in **a** and **b**), or with 50 μ g ml⁻¹ MB4B4 (lanes 5–7 in **c** and **d**). They were lysed in buffer containing Brij-96 as described¹⁵. After immunoprecipitation with phosphotyrosine mAb PY72 (gift from G. Mills and B. Sefton), samples were reduced and loaded onto 10% SDS–polyacrylamide gels, separated, and transferred electrophoretically to PVDF membranes. **a**, **c**, Western blots obtained with phosphotyrosine mAb 4G10 (gift from B. Druker). **b** and **d**, Same blots as in **a** and **c**, respectively, after being stripped of 4G10. Membranes were probed with mAbs to PLC- γ_1 (Upstate Biotech), the position of which is indicated. In the presence of MB23G2, substantially more tyrosine-phosphorylated PLC- γ_1 could be identified than in its absence, whereas MB4B did not alter the amount of PLC- γ_1 immunoprecipitated. We confirmed this using the same stimulation and immunoprecipitation with mAbs to PLC- γ_1 , followed by immunoblotting with phosphotyrosine mAb 4G10.

termed OX1 (raised against pan-CD45) was ineffective at prolonging graft survival. The lack of success with these antibodies can be explained in the light of our previous observations that only CD45RB inhibits allogeneic mixed lymphocyte reaction, whereas an antibody against pan-CD45 is ineffective⁷. The immunosuppression achieved may also be applicable in the prevention and treatment of transplant rejection in humans. □

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Neutrophil rolling altered by inhibition of L-selectin shedding *in vitro*

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THE L-selectin adhesion molecule is involved in guiding leukocytes to sites of inflammation¹. L-selectin is cleaved by an unusual proteolytic activity at a membrane-proximal site resulting in rapid shedding from the cell surface^{2–7}. Although it has been demonstrated that L-selectin mediates, in part, the early event of leukocyte rolling under hydrodynamic flow^{8–10}, the contribution of shedding to L-selectin function has remained unknown. Here we show that hydroxamic acid-based metalloprotease inhibitors block L-selectin downregulation from the cell surface of stimulated neutrophils, without affecting Mac-1 mobilization or general neutrophil activation, and inhibit cleavage of L-selectin in a cell-free system. Unexpectedly, the hydroxamic acid-based inhibitors reduced neutrophil rolling velocity under hydrodynamic flow, resulting in increased neutrophil accumulation. These results suggest that L-selectin is cleaved in seconds—much faster than previously suspected—during the process of rolling under hydrodynamic flow, and that shedding of L-selectin may contribute significantly to the velocity of leukocyte rolling. L-selectin shedding during rolling interactions may be physiologically important for limiting leukocyte aggregation and accumulation at sites of inflammation.

We constructed an L-selectin-alkaline phosphatase (L-AP) reporter to screen for inhibitors of L-selectin shedding and found that KD-IX-73-4 (Fig. 1a), a hydroxamic acid-based peptide inhibitor of matrix metalloproteases¹¹, prevented release of the L-AP reporter³¹. KD-IX-73-4 inhibited the downregulation of L-selectin from neutrophils stimulated with either 10^{−7} M formyl-methionylleucylphenylalanine (fMLP) (Fig. 1b) or 100 ng ml^{−1} 12-O-tetradecanoylphorbol-13-acetate (TPA). The inhibition of L-selectin shedding was dose-dependent with a 50% inhibitory concentration (IC₅₀) of about 4 μM. The compound did not non-

specifically inhibit neutrophil activation, as it had no effect on Mac-1 upregulation (Fig. 1b), did not affect the characteristic shift in forward- and side-light scatter profiles of activated cells, and had no effect on zymosan-stimulated neutrophil oxidative burst (data not shown). The diastereomer KD-IX-73-5 was about 25-fold less active, with partial activity at 50 μg ml^{−1} and no significant activity at 1 μg ml^{−1} (Fig. 1b).

Cleavage of L-selectin results in the formation of a 6K cleavage fragment which includes the transmembrane and cytoplasmic domains of L-selectin⁵. The KD-IX-73-4 prevented the disappearance of the intact 90K cell surface form of L-selectin (Fig. 2a, upper arrow, compare lanes 3, 4 and 5 with lane 2) and prevented the formation of the 6K transmembrane cleavage product (Fig. 2a, lower arrow) on neutrophils activated with either TPA (Fig. 2a) or fMLP (data not shown), in a dose-dependent manner. Again the less active diastereomer KD-IX-73-5 had partial activity at 50 μg ml^{−1} (Fig. 2a, lane 6) and no appreciable activity at 2 μg ml^{−1} (Fig. 2a, lane 8). A trapping enzyme-linked immunosorbent assay (ELISA) for soluble L-selectin showed inhibition of soluble L-selectin released by activated neutrophils treated with KD-IX-73-4 (not shown). KD-IV-86-1 and E559, two related compounds (Fig. 1 legend), had similar effects on inhibiting L-selectin shedding. A putative L-selectin secretase has been partially purified from Raji cell membrane preparations (J.M.F. et al., manuscript in preparation). An L-selectin-FLAG substrate (see Fig. 2 legend) was purified and incubated in a cell-free system with fractions containing secretase activity, yielding the expected 6K cleavage fragment (Fig. 2b, lane 1 versus lane 2). Like L-selectin proteolysis on intact cells, cleavage of the L-selectin substrate in the cell-free assay was not inhibited by a broad panel of common protease inhibitors, including 5 mM EDTA (data not shown). However, KD-IX-73-4 inhibited cleavage of the L-selectin substrate by the putative secretase activity, with a potency similar to that seen with whole cells (Fig. 2b, lanes 3–6). These data suggest that KD-IX-73-4 acts specifically to inhibit L-selectin proteolysis.

We next tested whether inhibition of L-selectin proteolysis would alter neutrophil rolling behaviour *in vitro* under hydrodynamic flow. To isolate the L-selectin-dependent component of neutrophil rolling interactions, we examined neutrophil rolling on purified MECA-79 antigen, isolated from inflamed human tonsil. CD34 is the main component of the MECA-79 antigen from human tonsil¹², and a known L-selectin ligand expressed on both lymph node and systemic vascular endothelium¹³. The human tonsil MECA-79 antigen supports both neutrophil and lymphocyte rolling, and monoclonal antibody directed against L-selectin completely inhibits neutrophil rolling interactions on MECA-79 antigen¹⁴. Treatment of the neutrophils with 10–25 μg ml^{−1} KD-IX-73-4 or E559 decreased leukocyte rolling velocity and increased leukocyte attachment per mm² at a wall shear stress ranging from 2 to 4 dynes cm^{−2}. The mean velocity of rolling neutrophils at 2 dynes cm^{−2} decreased from 27.7 μm s^{−1} for control cells to 9.2 μm s^{−1} for neutrophils treated with KD-IX-73-4 (Fig. 3a). At a higher wall shear stress of 4 dynes cm^{−2}, the difference in

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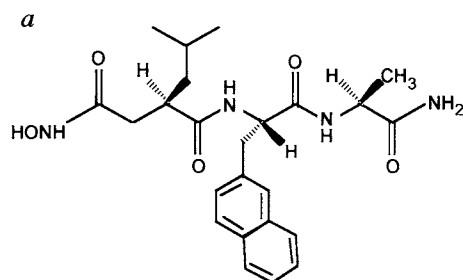
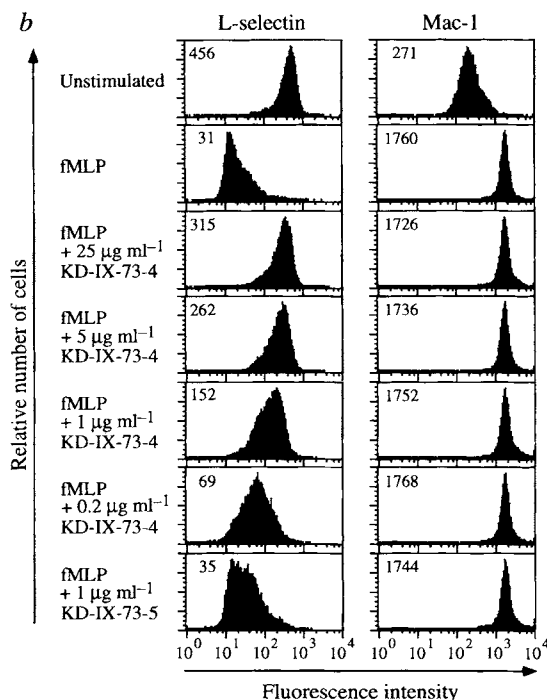


FIG. 1 Hydroxamic acid-based compounds inhibit L-selectin downregulation on leukocytes. **a**, Structure of the KD-IX-73-4 compound (*N*-L-(2-(hydroxyaminocarbonylmethyl)-4-methylpentanoyl)-L-3-(2'-naphthyl)-alanyl-L-alanine amide). KD-IX-73-4 and its diastereomer KD-IX-73-5 (*N*-D-(2-(hydroxyaminocarbonylmethyl)-4-methylpentanoyl)-L-3-(2'-naphthyl)-alanyl-L-alanine amide) were separated by reverse-phase HPLC. Two related compounds tested are KD-IV-86-1 (*N*-L-(2-(hydroxyaminocarbonylmethyl)-4-methylpentanoyl)-L-Trp-L-Ala amide) and E559 (*N*-R-(2-(hydroxyaminocarbonylmethyl)-4-methylpentanoyl)-L-Tyr-L-Ala (1,2-diaminoethyl)-biotin). **b**, KD-IX-73-4 inhibits downregulation of cell-surface expression of L-selectin. Freshly isolated neutrophils were preincubated for 10 min with various concentrations of KD-IX-73-4 or KD-IX-73-5, as indicated, and left unactivated (top panels) or activated for 15 min with 10^{-7} M fMLP (lower panels), as indicated. Neutrophils were stained for L-selectin expression with phycoerythrin-conjugated Leu-8 (left panels), for Mac-1 expression with phycoerythrin-conjugated Leu-15 (right panels), or stained with a control PE-conjugated mouse monoclonal antibody (MAb) (not shown), and analysed on a FACScan flow cytometer (Becton Dickinson). Mean fluorescence values are indicated in the upper left corner of each panel.



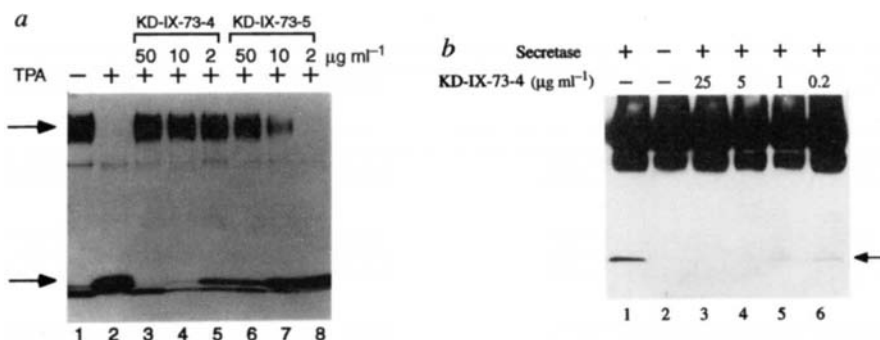
mean rolling velocity between control cells ($39.4 \mu\text{m s}^{-1}$) and treated cells ($25.6 \mu\text{m s}^{-1}$) was reduced, but still statistically significant ($P < 0.05$). Neutrophil attachment increased from $21.8 \pm 1.9 \text{ s.e.m. cells per mm}^2$ for control neutrophils to $94.0 \pm 3.9 \text{ s.e.m. cells per mm}^2$ for neutrophils treated with $25 \mu\text{g ml}^{-1}$ KD-IX-73-4 at a wall shear stress of 2 dynes cm^{-2} (Fig. 3b). In contrast, neutrophil rolling velocity on P-selectin was not significantly affected by treatment with KD-IX-73-4 at a

wall shear stress of 2 dynes cm^{-2} (Fig. 3c) or 4 dynes cm^{-2} (data not shown), indicating that the effects of KD-IX-73-4 are specific for L-selectin-mediated rolling interactions.

Single-cell analysis of time versus rolling distance revealed that control neutrophils (Fig. 4, upper panel) exhibit primarily a fast rolling phenotype on MECA-79 antigen (as shown by the steep slope of the rolling profiles) disrupted by short periods of slow rolling (horizontal portions of the rolling profiles). In contrast,

FIG. 2 Hydroxamic acids inhibit proteolysis of L-selectin.

a, Western blot analysis of the 6K transmembrane cleavage product. Freshly isolated neutrophils were activated with 100 ng ml^{-1} TPA in the presence of inhibitor, as indicated. Upper arrow, position of the intact 90K cell-surface form of L-selectin. Lower arrow, position of the 6K transmembrane cleavage product (the band present just below the 6K cleavage product is observed in some but not most neutrophil preparations analysed to date, and its origin is presently unknown). **b**, Cleavage of L-selectin in a cell-free assay. Raji cell membrane proteins were solubilized and fractionated as described below. Purified L-selectin containing a C-terminal FLAG epitope was immobilized on agarose beads by ConA lectin and incubated with buffer control (lane 2) or with the secretase partially purified from Raji membrane proteins (lanes 1, 3–6). Samples were treated with KD-IX-73-4, as indicated. Generation of the 6K cleavage product was assessed by western blot analysis. **METHODS.** Peripheral blood neutrophils were isolated by dextran sedimentation and Ficoll centrifugation, as previously described¹⁰. Neutrophil cell lysates were immunoprecipitated with a Sepharose-bound CA21 MAb directed toward the cytoplasmic domain of L-selectin⁵. The CA21-Sepharose was extensively washed, and specifically bound material was eluted with SDS sample buffer. Samples were resolved on a 10–20% tricine SDS-polyacrylamide gel (Novex) under non-reducing conditions and electrotransferred to a nitrocellulose membrane. The membrane was blocked in a 2% BSA-TBST solution, probed with biotinylated CA21 and washed. Following an incubation with a streptavidin-horseradish peroxidase conjugate, the membrane was washed and visualized by addition of ECL chemiluminescent substrate and exposure to film, as per manufacturer's instructions (Amersham). Raji cells were disrupted by sonication, and



membranes were washed with 0.5 M NaCl . Nuclei were removed by low-speed centrifugation, and the membrane fractions were solubilized in 25 mM HEPES containing 0.5% Triton X-100 and a cocktail of protease inhibitors (Boehringer Mannheim). Membrane proteins were purified over successive columns of Q anion exchange beads (Pharmacia), hydroxyapatite beads, Mono Q anion exchange beads (Pharmacia), and Mini-S cation exchange beads (Pharmacia). L-selectin protease activity was followed by cleavage of purified L-selectin substrate to yield the expected 6K cleavage fragment in a cell-free assay (J.M.F., manuscript in preparation). Briefly, an L-selectin construct, in which the C-terminal 8 amino acids of the cytoplasmic domain are replaced by an 8 amino-acid FLAG-epitope (Kodak), was expressed, purified to homogeneity, and then immobilized to agarose beads by ConA lectin. A sample ($5\text{--}10 \mu\text{l}$) of the L-selectin-bead slurry was incubated with secretase purified from Raji membrane proteins. Generation of the 6K cleavage fragment was analysed by western blot analysis with the anti-FLAG MAb. Similar results were obtained with wild-type L-selectin immobilized by an anti-L-selectin ectodomain MAb and detected with a MAb directed against the cytoplasmic tail of L-selectin (data not shown).

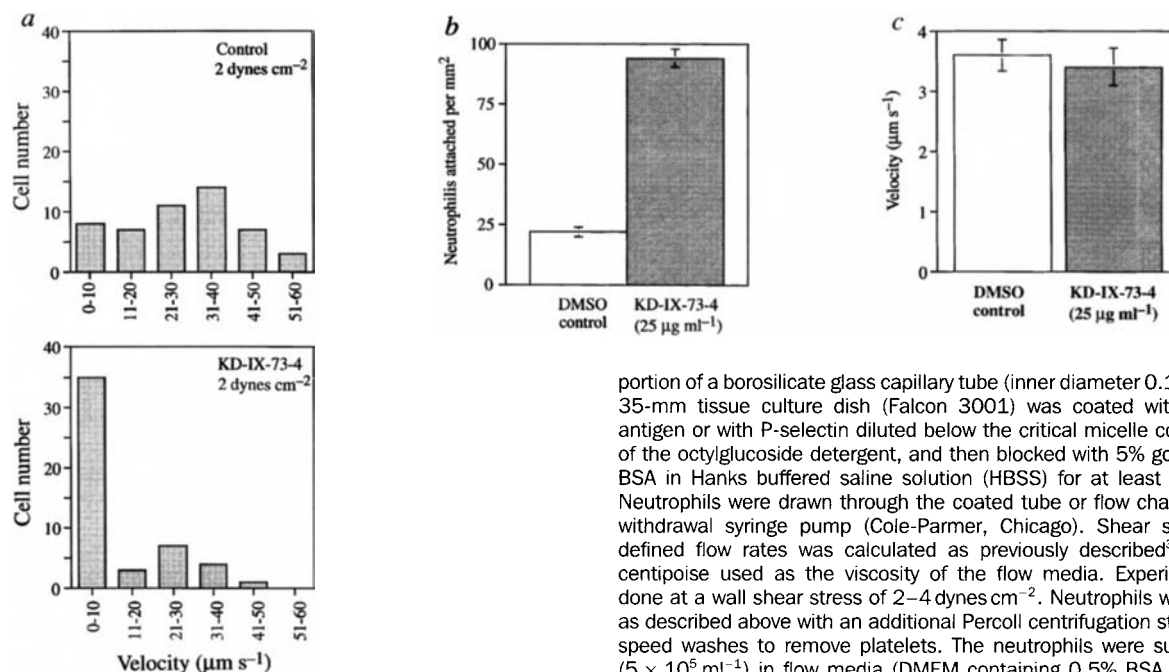


FIG. 3 Hydroxamic-acid-based compounds affect neutrophil rolling under hydrodynamic flow on MECA-79 antigen but not on P-selectin. **a**, KD-IX-73-4 decreases neutrophil rolling velocity on MECA-79 antigen. Neutrophils were treated with 25 μg ml⁻¹ KD-IX-73-4 or with dimethylsulphoxide (DMSO) carrier, as indicated, and then allowed to establish a rolling interaction on MECA-79 antigen at a wall shear stress of 2 dynes cm⁻². Rolling velocities of 50 individual neutrophils were analysed in both control and treated groups. Data are expressed as a population histogram. The mean velocity of KD-IX-73-4-treated cells (9.2 μm s⁻¹) versus control cells (27.7 μm s⁻¹) is statistically significant ($P \leq 0.001$; Wilcoxon 2-sample test). **b**, KD-IX-73-4 increases neutrophil attachment on MECA-79 antigen. Neutrophil attachment was calculated by analysing video frames every 15 s over a period of 2 min and averaging the total number of neutrophils bound per mm². **c**, KD-IX-73-4 does not affect neutrophil rolling velocity on P-selectin. Neutrophils were treated with KD-IX-73-4 or DMSO, as described above, and allowed to establish a rolling interaction on P-selectin at a wall shear stress of 2 dynes cm⁻². Rolling velocity of 50 individual neutrophils were analysed in both control and treated groups.

METHODS. Neutrophil rolling under hydrodynamic shear was done with a capillary tube flow system as previously described¹⁸ with the modification that neutrophils were analysed in a single pass rather than by continuous recirculation, or with a parallel plate flow chamber (Glycotech), as previously described¹⁴. Purified MECA-79 antigen was isolated from human tonsil. P-selectin was purified from human platelets with the WAPS 12.2 MAb. A

portion of a borosilicate glass capillary tube (inner diameter 0.112 cm) or a 35-mm tissue culture dish (Falcon 3001) was coated with MECA-79 antigen or with P-selectin diluted below the critical micelle concentration of the octylglucoside detergent, and then blocked with 5% goat serum or BSA in Hanks buffered saline solution (HBSS) for at least 1 h at 4 °C. Neutrophils were drawn through the coated tube or flow chamber with a withdrawal syringe pump (Cole-Parmer, Chicago). Shear stress under defined flow rates was calculated as previously described³⁰, with 1.5 centipoise used as the viscosity of the flow media. Experiments were done at a wall shear stress of 2–4 dynes cm⁻². Neutrophils were isolated as described above with an additional Percoll centrifugation step and low-speed washes to remove platelets. The neutrophils were suspended at (5×10^5 ml⁻¹) in flow media (DMEM containing 0.5% BSA and 25 mM HEPES). In all experiments using MECA-79 antigen, anti-CD18 MAb (R15.7) and the anti-P-selectin MAb (WAPS-12.2) were included to eliminate the contribution of CD18-mediated stable arrest and P-selectin-mediated tethering to residual platelets, respectively. Rolling on P-selectin-coated surfaces was performed in the presence of anti-CD18 MAb. Neutrophils were allowed to interact with the MECA-79 antigen-coated surfaces for 5 min at a specified shear stress in order to equilibrate tethering before analysis. Neutrophil rolling was visualized with a Nikon inverted microscope equipped with a Hoffman modulation contrast condenser (Modulation Optics Inc., Greenvale) using a $\times 20$ lens, and recorded to VHS video tape for later analysis with NIH image analysis software on a Macintosh Quadra 950. Neutrophils rolled on the MECA-79 antigen- and P-selectin-coated surfaces but not on uncoated surfaces. All interactions of neutrophils with MECA-79 antigen or P-selectin were eliminated by the addition of anti-L-selectin MAb (DREG-200) or anti-P-selectin MAb (WAPS 12.2), respectively. To test the effects of the hydroxamic-acid compounds, neutrophils (5×10^5 ml⁻¹) were pretreated with KD-IX-73-4 or E559 at 10–25 μg ml⁻¹ in flow media for 5 min at room temperature. Control neutrophils were treated with an equivalent amount of DMSO used to solubilize the hydroxamic-acid derivatives. In some experiments, the compounds were added to the leukocyte reservoir after establishment of a rolling interaction. Within each experiment, the same field of the capillary tube or flow chamber was analysed for both treated and untreated neutrophils. The order in which cells were analysed—treated versus untreated groups—did not affect the results.

neutrophils treated with hydroxamic acid-based compounds showed increased periods of slow rolling or transient stopping, as shown by the extended horizontal portions of the line graphs (Fig. 4, lower panel). The addition of antibody against CD18 to eliminate integrin-mediated arrest had no effect on control or KD-IX-73-4-treated neutrophil rolling on MECA-79 antigen. In contrast, addition of the anti-L-selectin antibody DREG-200 to KD-IX-73-4-treated neutrophils inhibited all interactions with MECA-79 antigen (data not shown).

Crosslinking of L-selectin with anti-L-selectin antibody induces shedding¹⁵. It was previously postulated that in a physiological setting, neutrophil rolling may result in local crosslinking of L-selectin on microvilli tips and cause selective shedding of those L-selectin molecules engaged in ligand binding¹⁵. The impact of the hydroxamic acid-based inhibitors on neutrophil rolling provides experimental evidence that L-selectin proteolysis can occur during the time course of neutrophil rolling interactions. Neutrophils are capable of maintaining a rolling interaction over an extended distance on MECA-79 antigen, suggesting that L-selectin proteolysis may be limited, perhaps to those L-selectin molecules bound to ligand. It is possible that binding of ligand under

hydrodynamic flow may provide a rapid signal to the putative protease or expose the cleavage site of L-selectin itself. The hydroxamic acid-based inhibitors have a substantial effect on the velocity of L-selectin-dependent, but not P-selectin-dependent, neutrophil rolling, suggesting that the effect of the inhibitor is specific for L-selectin-mediated interactions. However, we cannot formally exclude the possibility that the inhibitor may have unexpected actions, in addition to inhibiting L-selectin shedding, that may affect neutrophil rolling. L-selectin shedding, in addition to the extremely high 'off rates' for selectin-carbohydrate bonds¹⁶, may account in part for the fast rolling adhesions mediated by L-selectin. Leukocytes need to be able to scan efficiently the vasculature for sites of inflammation, and L-selectin shedding during the rolling process may enable leukocytes to detach rapidly if an appropriate chemokine signal is lacking. A general role in surveillance of vascular endothelium may account for the high concentrations of soluble L-selectin present in normal serum^{15,17}. Neutrophils express both L-selectin and a functional ligand for L-selectin^{18,19}. Another physiological role for ligation-dependent shedding may be to regulate L-selectin-dependent neutrophil-neutrophil interactions, which can lead to neutrophil

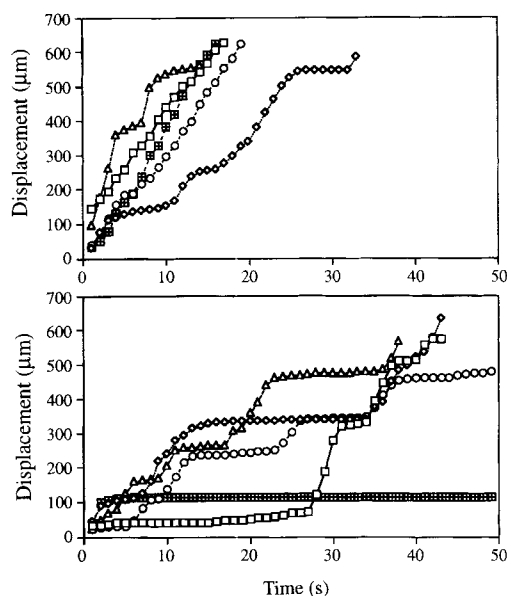


FIG. 4 Single-cell analysis of neutrophil rolling. Neutrophils were treated with DMSO carrier (upper panel) or with $25 \mu\text{g ml}^{-1}$ of the hydroxamic acid-based compound (lower panel) and then allowed to establish a rolling interaction at a wall shear stress of 3 dynes cm^{-2} on a MECA-79 antigen-coated borosilicate glass capillary tube. The rolling profile of distance (μm) versus time (s) was determined for 5 randomly selected cells in each group. Representative results from one of five independent experiments are shown.

accumulation under flow¹⁸ and neutrophil aggregation¹⁹. In contrast, the presence of an appropriate chemokine signal results in global shedding of L-selectin and engagement of the CD18 integrins, leading to transendothelial migration. Global shedding of L-selectin may aid transmigration or may be a protective mechanism to prevent further trafficking of activated neutrophils. Thus L-selectin shedding may occur through two mechanisms—receptor ligation and chemokine activation—with distinct physiological outcomes.

L-selectin is part of an emerging and diverse class of proteins, including tumour necrosis factor (TNF)^{20–22}, transforming growth factor- α (TGF- α)²³, β -amyloid precursor protein²⁴, angiotensin-converting enzyme²⁵, and interleukin-6R²⁶, whose cell-surface expression is regulated by activation-induced proteolysis at a membrane proximal site. Recently hydroxamic acid-based peptides have been demonstrated to inhibit activation-induced shedding of TNF^{20–22} and TNF receptor²⁷, suggesting that a related protease activity may be involved in the regulation of at least TNF and L-selectin. Both the membrane-bound and soluble forms of TNF and other receptors and growth factors are thought to be biologically active^{28,29}. The receptor ligation-induced shedding mechanism shown for L-selectin may have implications for these other biological systems as a cell contact-dependent, activation-independent means of rapidly processing growth factors, cytokines and receptors presented on the cell surface.

A shear threshold requirement for L-selectin-mediated adhesion has recently been described, and it has been speculated that the shear threshold may prevent adhesion under low flow conditions³². The shear threshold requirement and the ligation-dependent shedding of L-selectin are not mutually exclusive models for regulating neutrophil aggregation and accumulation. □

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Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis

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BINDING of Fas ligand or an agonistic anti-Fas antibody induces apoptosis in Fas-bearing cells¹. The interleukin-1 β -converting enzyme (ICE) is a cysteine protease² that is involved in apoptosis induced by various stimuli, including Fas-mediated apoptosis^{3–8}. Several ICE homologues have been identified, and these are subdivided into three groups (ICE-, CPP32- and Ich-1-like proteases)^{9–18}. We show here that specific inhibitors of ICE- or CPP32-like proteases can inhibit Fas-mediated apoptosis. Transient ICE-like activity was found in the cytosolic fraction of Fas-activated cells, whereas ICE-dependent, CPP32-like activity gradually accumulated in the cytosol. Cell lysates from mouse lymphoma supplemented with either recombinant ICE or CPP32 induced apoptosis of nuclei. The CPP32 inhibitor inhibited ICE- or CPP32-induced apoptosis in the cell-free system, whereas the ICE-inhibitor only inhibited ICE-induced apoptosis. Cell extracts from thymocytes from ICE-null mice induced apoptosis in the cell-free system when it was supplemented with CPP32. These results indicate that Fas sequentially activates ICE- and CPP32-like proteases, and that downstream CPP32, together with a component(s) in the cytoplasm, causes apoptosis of nuclei.

Among members of the ICE family, ICE and CPP32 have different substrate specificities^{16,19}. We examined the involvement of ICE and CPP32 in Fas-mediated apoptosis using two tetrapeptides: acetyl-Tyr-Val-Ala-Asp-CHO (Ac-YVAD-cho) and

FIG. 1 Effect of Ac-YVAD·cho or Ac-DEVD·cho on Fas-mediated apoptosis. *a*, Mouse W4 cells³⁰ (2.5×10^4 cells) in 0.1 ml were pre-incubated for 1 h with various concentrations of either Ac-YVAD·cho (triangles) or Ac-DEVD·cho (circles) (Peptides Inst. Osaka). Cells were then incubated at 37 °C for 4 h in the presence (open symbols) or absence (filled symbols) of 300 ng ml⁻¹ anti-Fas antibody (Jo2) (ref. 30). Percentages of viable cells were determined by Trypan-blue exclusion²⁴. *b*, After preincubation at 37 °C for 1 h without (crosses) or with 300 μ M Ac-YVAD·cho (triangles) or Ac-DEVD·cho (circles), W4 cells (2.5×10^4 cells) were incubated at 37 °C for various periods in the presence of 300 ng ml⁻¹ anti-Fas antibody. Cell viability was determined as for *a*.

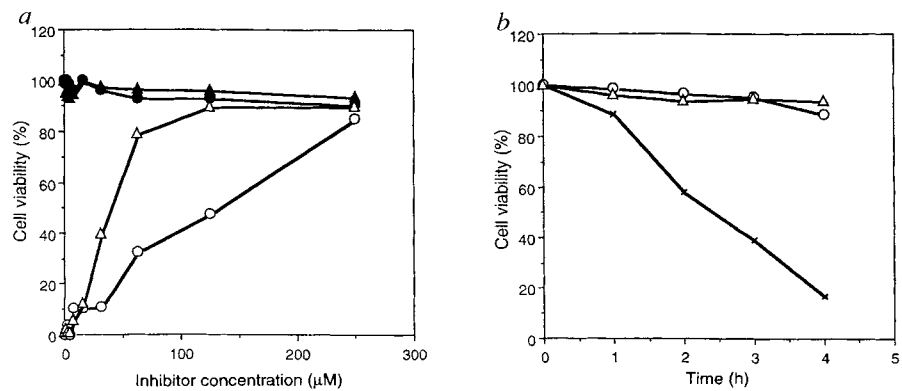
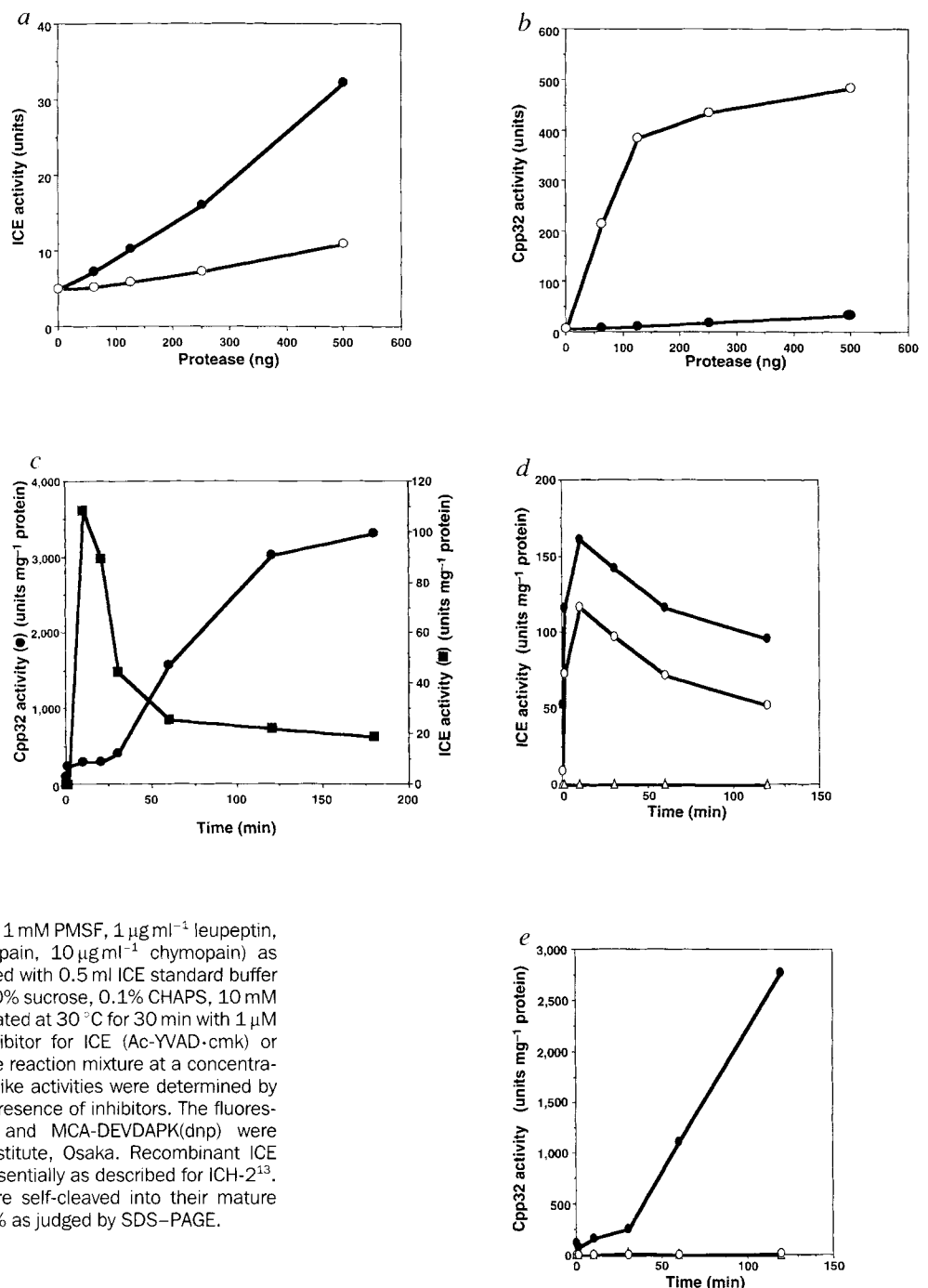


FIG. 2 Sequential activation of ICE-like and CPP32-like proteases during Fas-induced apoptosis. *a* and *b*, The fluorescent substrate, either MCA-YVADAPK(dnp) (*a*), or MCA-DEVDAPK(dnp) (*b*), was incubated at 30 °C for 30 min with various amounts of the recombinant ICE (filled circles) or CPP32 β (open circles), and the fluorescence of the cleaved substrates was determined using a spectrofluorometer set at an excitation wavelength of 325 nm and an emission wavelength of 392 nm. *c*, Cytosolic extracts were prepared from W4 cells (1×10^7 cells) which were treated at 37 °C with 300 ng ml⁻¹ of anti-Fas antibody for various times. ICE- and CPP32-like activity in the lysates (36 μ g protein) was then determined using the fluorescent substrates, MCA-YVADAPK(dnp) (squares) or MCA-DEVDAPK(dnp) (circles), respectively. *d* and *e*, W4 cells were treated for various times with anti-Fas antibody alone (filled circles) or in the presence of either 300 μ M Ac-YVAD·cmk (open triangles) or Ac-DEVD·cho (open circles). The ICE-like (*d*) or CPP32-like activity (*e*) in the cell extract (36 μ g protein) was assayed; one unit corresponds to the activity that cleaves 1 μ mol of the respective fluorescent substrate at 30 °C in 30 min.



METHODS. Cytosolic extracts were prepared by repeated freezing and thawing of cells in 100 μ l extraction buffer (50 mM PIPES-NaOH, pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM DTT, 20 μ M cytochalasin B, 1 mM PMSF, 1 μ g ml⁻¹ leupeptin, 1 μ g ml⁻¹ pepstatin A, 50 μ g ml⁻¹ antipain, 10 μ g ml⁻¹ chymopain) as described²¹. Cell lysates were then diluted with 0.5 ml ICE standard buffer (100 mM HEPES-KOH buffer, pH 7.5, 10% sucrose, 0.1% CHAPS, 10 mM DTT, 0.1 mg ml⁻¹ ovalbumin), and incubated at 30 °C for 30 min with 1 μ M fluorescent substrate. The specific inhibitor for ICE (Ac-YVAD·cmk) or CPP32 (Ac-DEVD·cho) was added to the reaction mixture at a concentration of 1 μ M. Specific ICE- and CPP32-like activities were determined by subtracting the values obtained in the presence of inhibitors. The fluorescent substrates, MCA-YVADAPK(dnp) and MCA-DEVDAPK(dnp) were custom-synthesized at the Peptides Institute, Osaka. Recombinant ICE and CPP32 β were prepared in *E. coli* essentially as described for ICH-2¹³. The recombinant ICE and CPP32 β were self-cleaved into their mature forms; their purities were more than 80% as judged by SDS-PAGE.

acetyl-Asp-Glu-Val-Asp-CHO (Ac-DEVD·cho), which are specific inhibitors of ICE and CPP32, respectively^{16,19}. As shown in Fig. 1, mouse WR19L transformant cells (W4), which constitutively express mouse Fas, were killed within four hours by an agonistic anti-Fas antibody. When W4 cells were preincubated with either Ac-YVAD·cho or Ac-DEVD·cho, Fas-induced apoptosis was inhibited. Kinetic analysis of the death process in the presence of Ac-YVAD·cho or Ac-DEVD·cho confirmed this inhibitory effect (Fig. 1b).

The ICE- and CPP32-like activities in Fas-activated cell extracts were then measured. To distinguish between the two activities, we prepared two peptides, MCA-YVADAPK(dnp) and MCA-DEVDAPK(dnp), in which specific peptides were coupled to the highly fluorescent (7-methoxycoumarin-4-yl)acetyl group (MCA) and its quenching 2,4-dinitrophenyl (dnp) group. As shown in Fig. 2a, recombinant ICE cleaved MCA-YVADAPK(dnp), but DPP32 β was only very weakly active on the substrate. On the other hand, CPP32 β efficiently cleaved MCA-DEVDAPK(dnp), whereas ICE had little activity on this substrate (Fig. 2b). The specific activities of the recombinant ICE for MCA-YVADAPK(dnp) and CPP32 for MCA-DEVDAPK(dnp) were 64 and 3,300 units per μ g protein, respectively.

W4 cells were treated for various periods with the anti-Fas antibody, after which the ICE- and CPP32-like activities in the

cytosolic extracts were determined using fluorescent substrates. Extracts from untreated W4 cells did not show ICE-like activity (Fig. 2c). Fas activation induced transient ICE-like activity with a peak at 10 min (110 units per mg protein). The cytosol of untreated W4 cells showed a low level of CPP32-like activity, which was gradually increased up to 3,300 units per mg protein by Fas activation. If we assume that the ICE- and CPP32-like proteases in the cytosol have specific activities similar to recombinant ICE and CPP32, the results in Fig. 2c suggest that ICE- and CPP32-like proteases were comparably activated but with different kinetics during Fas-mediated apoptosis. The appearance of ICE-like activity before CPP32-like activity suggested that the activation of the CPP32-like protease might depend on the presence of the ICE-like protease. To examine this possibility, we treated W4 cells with anti-Fas antibody in the presence of Ac-YVAD·chloromethylketone (cmk) or Ac-DEVD·cho, and determined the ICE- and CPP32-like activities in the cytosol. We found that Ac-YVAD·cmk completely inhibited the generation of ICE-like activity (Fig. 2d), but that Ac-DEVD·cho had only a small effect on it. In contrast, both Ac-YVAD·cmk and Ac-DEVD·cho completely inhibited Fas-induced generation of CPP32-like activity (Fig. 2e). These results indicate that the production of CPP32-like activity during Fas-mediated apoptosis depends on the previous presence of ICE-like activity.

Cytosolic extracts from Fas-activated cells cause apoptosis in the nuclei from living cells²⁰⁻²². These extracts contained active ICE and CPP32-like proteases (Fig. 2). Using reverse transcription with the polymerase chain reaction (RT-PCR), together with primers specific for ICE or CPP32 messenger RNA and northern hybridization analysis with ICE or CPP32 complementary DNA probes, we found that ICE and CPP32 were expressed in W4 cells (data not shown). We therefore tested whether either ICE or CPP32 could induce apoptosis in the cell-free system. When nuclei from mouse liver were incubated with either recombinant ICE or CPP32 β alone, they did not undergo apoptosis (Fig. 3a). However, cell lysates from unstimulated W4 cells, supplemented by ICE or CPP32 β , induced DNA degradation; CPP32 β was more potent than ICE in inducing apoptosis (Fig. 3b). This DNA degradation was accompanied by the morphological change in the nuclei that is typical of apoptosis (Fig. 3c). Trypsin or proteinase K did not induce apoptosis either alone or with cell lysates (data not shown).

We next investigated whether ICE-induced apoptosis in a cell-free system is mediated by CPP32-like activity. As shown in Fig. 4, when the lysates from W4 cells were incubated with ICE, CPP32-like activity was generated in the lysates (lane 1). Ac-YVAD·cho inhibited ICE activity, the production of CPP32-like protease in the cell lysates, and ICE-induced apoptosis of nuclei (lane 2). On the other hand, Ac-DEVD·cho did not inhibit ICE, but did inhibit ICE-induced production of CPP32-like activity in the cell lysates and apoptosis in the nuclei (lane 3). CPP32 β did not generate ICE-like activity (lane 4), and CPP32-induced apoptosis was inhibited by Ac-DEVD·cho but not Ac-YVAD·cho (lanes 5 and 6). These results suggest that ICE first activates CPP32-like protease in the cell lysates, and that CPP32-like protease then causes apoptosis in nuclei together with a component(s) in the lysates. Thymocytes from mice lacking ICE are resistant against Fas-mediated apoptosis⁸. When cell lysates of thymocytes from ICE-null mice²³ were supplemented with recombinant CPP32 β , they induced apoptosis in nuclei as efficiently as lysates from wild-type mice (Fig. 4d). These results confirmed that CPP32-induced apoptosis does not require ICE, and suggested that the defect in Fas-mediated apoptosis of thymocytes in ICE-null mice is due to the failure of ICE-dependent activation of CPP32.

Three models may explain the contribution of at least seven ICE members in apoptosis: (1) each member independently induces apoptosis; (2) members of the family sequentially activate other members; or (3) at least two members are required in parallel to induce apoptosis. Here we have shown that Fas induces a sequential activation of ICE-like and CPP32-like proteases, and

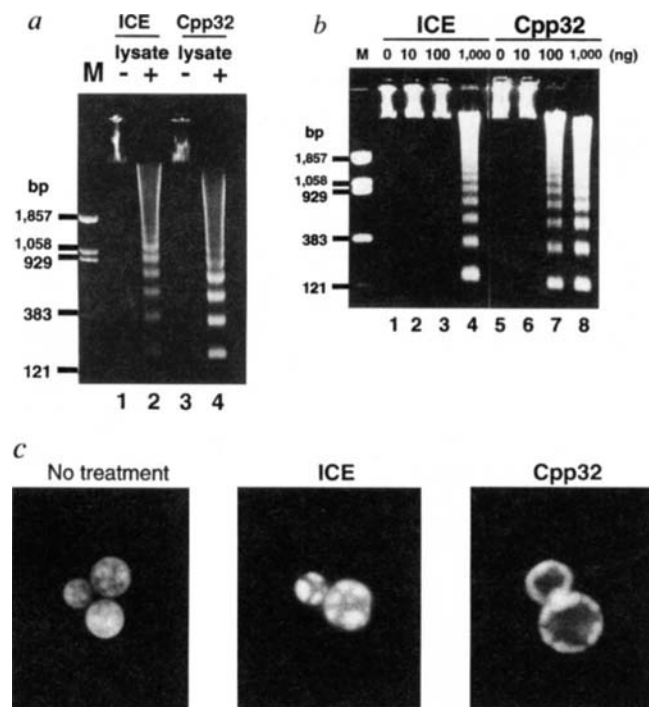
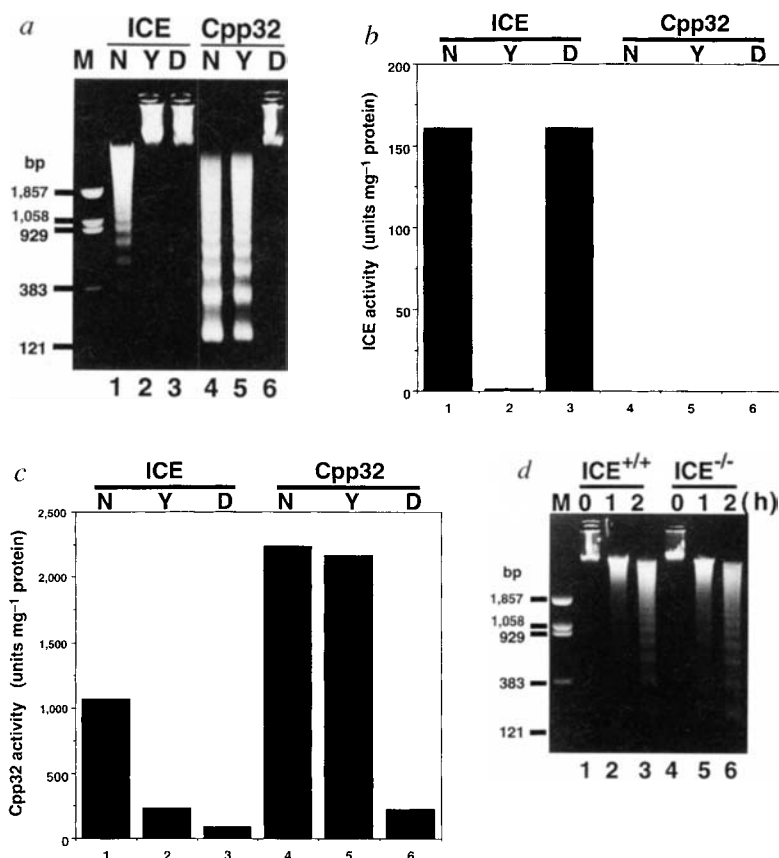


FIG. 3 Apoptosis induced by recombinant ICE or CPP32 β . a, Nuclei from mouse liver²¹ were incubated with recombinant human ICE (3 μ g) or CPP32 β (100 ng) in the absence or presence of cytosolic extracts (140 μ g) from unstimulated W4 cells. After incubation, nuclei were collected by centrifugation, and the chromosomal DNA analysed by electrophoresis on a 1.5% agarose gel. b, Nuclei were incubated with the indicated amounts of either ICE or CPP32 β combined with the cell lysates (140 μ g) from unstimulated W4 cells and the chromosomal DNA analysed. c, Nuclei from mouse liver were incubated with extracts (140 μ g) from unstimulated W4 cells (left panel), or with cell extracts supplemented with either 3 μ g recombinant ICE (middle) or 200 ng of CPP32 β (right). After incubation, nuclei were stained with 10 μ g ml⁻¹ of 4',6'-diamidino-2-phenylindole (DAPI), and observed under a fluorescence microscope with a UV-2A combination filter (Nikon OPTIPHOT).

METHODS. *In vitro* apoptosis was carried out essentially as described²¹. In brief, 3×10^6 nuclei from mouse liver were incubated at 37 °C for 3 h in 27 μ l of R buffer (10 mM HEPES-KOH, pH 7.0, 40 mM β -glycerophosphate, 50 mM NaCl, 2 mM MgCl₂, 5 mM EGTA, 1 mM DTT, 2 mM ATP, 10 mM creatine phosphate, 50 μ g ml⁻¹ creatine kinase and 0.2 mg ml⁻¹ BSA).

FIG. 4 CPP32 β -induced apoptosis does not apparently require ICE. **a–c**, Cell lysates from unstimulated W4 cells (140 μ g) were incubated at room temperature for 15 min with no additions (N) (lanes 1 and 4) or in the presence of either 180 nM Ac-YVAD-cho (Y) (lanes 2 and 5) or 72 nM Ac-DEVD-cho (D) (lanes 3 and 6) in 24 μ l of R buffer. Recombinant ICE (2.5 μ g) (lanes 1–3) or CPP32 β (200 ng) (lanes 4–6) was added to the reaction mixture and incubated at room temperatures for 15 min. Nuclei (3×10^6 in 3 μ l) were then added to the mixture, and further incubated at 37 °C for 3 h. Using 6- μ l aliquots, chromosomal DNA from the nuclei was analysed by electrophoresis on a 1.5% agarose gel (**a**), 2- μ l aliquots were used to assay ICE-like (**b**) and CPP32-like (**c**) activities with 1 μ M MCA-YVADAPK(dnp) or MCA-DEVDAPK(dnp) as substrate. **d**, Cell lysates were prepared from wild-type (ICE $^{+/+}$) or ICE-null mice (ICE $^{-/-}$) as described²¹. Nuclei (3×10^6) were incubated for the indicated periods at 37 °C in a final volume of 30 μ l with the cell lysates (25 μ g), which were supplemented with 20 ng recombinant CPP32 β . Chromosomal DNA was analysed as for **a**.



that downstream CPP32 is sufficient to cause apoptotic DNA degradation in nuclei together with a component(s) in the cytoplasm. These results support the second model. However the ICE-like proteases seem to be redundant, because, although the thymocytes from ICE-null mice show defective Fas-mediated apoptosis⁸, the mice did not show the lymphoproliferation phenotype seen in mice carrying the loss-of-function mutation of Fas or Fas ligand¹. Whether or not CPP32-like activity is also mediated by redundant proteases remains to be investigated.

Fas-mediated apoptosis proceeds without RNA or protein synthesis^{24–26}, indicating that the appearance of ICE- or CPP32-like activity is a post-translational activation of these proteases. It is likely that ICE-like and CPP32-like proteases are sequentially cleaved to become active forms. If so, why is the ICE-like protease only transiently activated? It is possible that mammalian cells contain CrmA- or p35-like inhibitors of ICE^{27,28}. The signal from Fas may transiently stimulate the dissociation of such inhibitors from the mature ICE-like protease or, alternatively, ICE activa-

tion might be followed by the activation of a different protease that cleaves ICE in order to inactivate it. CPP32-like activity gradually accumulates in the cytosol of Fas-activated cells. Although CPP32 can be directly cleaved by ICE¹⁷, it is not yet clear whether the ICE-like protease directly cleaves the CPP32-like protease, or whether other proteins acting between the two could be involved during Fas-induced apoptosis. Active CPP32 alone could not induce apoptosis in a cell-free system, but it caused apoptosis in nuclei supplemented with cell lysates. The requirement of the cytoplasmic fraction to induce apoptosis suggests that one or more additional components in the cytoplasm help CPP32 to enter the nucleus, where it cleaves various substrates such as poly(ADP) ribose polymerase²⁹. But it is also possible that the actual substrate for CPP32 that causes DNA degradation is in the cytoplasm, and that the activated substrate goes into the nucleus. In any event, our *in vitro* system of apoptosis using recombinant ICE or CPP32 will be useful in determining the apoptotic pathway downstream of CPP32. □

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Structure of the UmuD' protein and its regulation in response to DNA damage

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For life to be sustained, mistakes in DNA repair must be tolerated when damage obscures the genetic information. In bacteria such as *Escherichia coli*, DNA damage elicits the well regulated 'SOS response' (reviewed in ref. 1). For the extreme case of damage that cannot be repaired by conventional enzymes, there are proteins that allow the replication of DNA through such lesions, but with a reduction in the fidelity of replication². Essential proteins in this mutagenic process are RecA, DNA polymerase III, UmuD, UmuD' and UmuC (*umu*: UV mutagenesis)¹⁻³. Regulation of this response involves a RecA-mediated self-cleavage of UmuD to produce UmuD'. To understand this system in more detail, we have determined the crystal structure of the *E. coli* UmuD' mutagenesis protein at 2.5 Å resolution. Globular heads folded in an unusual β -structure associate to form molecular dimers, and extended amino-terminal tails associate to produce crystallized filaments. The structure provides insight into the mechanism of the self-cleavage reaction that UmuD-like proteins undergo as part of the global SOS response⁴⁻⁸.

UmuD' was purified and crystallized as described elsewhere⁹ and summarized in Table 1. The structure was determined by the multiwavelength anomalous diffraction (MAD) method¹⁰ from the selenomethionyl protein¹¹. To avoid an exposed site thought to interfere with crystallization of the selenomethionyl analogue, we used standard polymerase chain reaction (PCR) techniques to replace the methionine residue with a threonine residue at position 138⁹. Crystals grown from selenomethionine-substituted UmuD' (M138T) were frozen at 100 K and used for MAD measurements at the Brookhaven National Synchrotron Light Source (NSLS). Data from an initial four-wavelength experiment were used to find the selenium sites and to estimate phases for a 3.0-Å resolution MAD map in which the polypeptide chain was traced. Subsequently, better data extending to 2.5-Å Bragg spacings were measured in a three-wavelength experiment. The model was completed with these MAD data, which were also used to refine the model to an *R*-value of 21% (Table 1).

UmuD' adopts an unusual and apparently unique fold (Fig. 1a, b). The two UmuD' chains in the crystallographic asymmetric unit have essentially identical structures. Each protomeric unit has a globular head (residues 50–135) plus an extended N-terminal tail and a short carboxy-terminal extension. The globular domain is an antiparallel β -structure composed of a succession of highly curved β -hairpins, β_1 – β_2 , β_3 – β_4 and β_5 – β_6 , followed by a long terminal strand, β_7 . Only the segment connecting β_2 to β_3 is not a tight turn. The strands are organized into three β -sheets (β_1 – β_2 – β_7 , β_3 – β_4 – β_5 – β_6 and β_3 – β_4 – β_7) to form a three-layer structure with two separate hydrophobic cores. The β_1 – β_2 loop includes a segment of 3₁₀ helix that makes contact with the β_5 – β_6 loop to enclose one of the core segments. The N-terminal extension includes a two-turn α -helix. Residues 25–31 and 138–139 at the termini are disordered in the crystals, and there is little side-chain density for residues 32–36 except for Tyr 33, which is well defined.

A structure-based amino-acid sequence alignment of UmuD'

with three proteins from the homologous family strongly suggests that they adopt the same fold (Fig. 1c). UmuD', LexA and λ cI all form dimers in solution, and we see UmuD' as a dimer in the crystal structure as well. The UmuD' dimer interface is primarily hydrophobic and consists in part of residues Tyr 52, Val 54, Ile 87, Phe 94 and Phe 128, with the Phe 94 residues (one from each protomer) being stacked on top of each other (Fig. 2a). There is also a salt bridge between the conserved residue Glu 93 and Lys 55 of the dimer mate on both sides of the interface. Although Lys 55 is not absolutely conserved, the charge is conserved in all the UmuD'-like proteins (but not in the repressor proteins). About 1,100 Å² of accessible surface area is buried when two protomers form a molecular dimer. A filament is formed in the crystal structure by interactions at the N and C termini of the protein (Fig. 2b). Genetic and biochemical experiments (manuscript in preparation) support the biological relevance of this filament in the mutagenic repair of DNA lesions.

The UmuD protein is regulated not only at the transcriptional level by the LexA repressor (like most other SOS proteins), but also at a post-translational level. UmuD is activated by a RecA-mediated self-cleavage reaction that removes 24 N-terminal residues, forming UmuD' (refs 6–8). The conserved residues Ser 60 and Lys 97 (Fig. 1c) have been implicated in the self-cleavage reaction that converts UmuD to the mutagenically active form of UmuD' (ref. 8). These residues are found at one end of a cleft in the structure with the Ser 60 O γ located within hydrogen bonding distance of the Lys 97 N ϵ (2.8 Å, Fig. 3a). Both Ser 60 and Lys 97 are buried with only 8% and 6% of their respective surfaces accessible to water. Conceivably, the cleft in which Ser 60 and Lys 97 are found is the binding site for the N terminus, ensuring the correct orientation of the scissile bond between residues Cys 24 and Gly 25. Supporting this, several mutations in UmuD, LexA and the λ cI proteins that affect the efficiency of the cleavage reaction are located along the sides of this cleft^{5,12-14}. Moreover, the self-cleavage reaction is believed to occur when the protein is monomeric. Much of the cleft is covered by the dimer interface (Fig. 3b), compatible with the hypothesis that these proteins are cleaved as monomers¹⁵.

Recently it has been suggested that this reaction is comparable to that of the β -lactamases^{1,16}. Indeed, structural similarities suggest to us that the self-cleavage reaction works in a similar fashion to the hydrolysis of penicillin by the TEM1 β -lactamase¹⁷. Superimposing the atoms important for catalysis from the TEM1 structure onto the UmuD' structure (the Ser 70 O γ on Ser 60 O γ and the Lys 73 N ϵ on Lys 97 N ϵ) allows one to place the Asn 132 O δ (TEM1) onto Val 96 O (UmuD') with an r.m.s. deviation in these atoms of 0.12 Å (Fig. 3c). We therefore believe that Ser 60 and Lys 97 are correctly positioned in the UmuD' catalytic site to perform the peptide bond cleavage that activates this protein. Many studies have shown that the UmuD-like proteins undergo autodigestion *in vitro* under alkaline conditions, suggesting that Lys 97 must be deprotonated for cleavage to proceed^{4,5,7}. The binding of RecA may change the environment around the active site sufficiently to lower the pK_a of Lys 97, enabling the reaction to occur at physiological conditions.

The N terminus of our model is about 50 Å away from the active site residue Ser 60. Although the model is missing residues 25–31, even if these residues were extended back after a reverse turn the N terminus would still be remote from the active site. It seems plausible that the UmuD conformation of this region is noticeably different from that of the UmuD' crystal structure. Evidence (manuscript in preparation) suggests that part of the 'activating' step in the maturation of UmuD to UmuD' is a conformational change in the N terminus, moving it away from the active site and into the position seen in the crystal structure. Thus, it appears that UmuD' may make very different interactions with the activated RecA–DNA filament than does UmuD.

The structure of UmuD' provides insight into its regulated role in DNA mutagenesis. There is evidence suggesting that the filament seen in the crystal structure is functionally relevant

TABLE 1 Statistics for data collection, MAD phase determination and refinement

Data collection Wavelength (Å)	Unique reflections*	Completeness (%)*	R_{sym} (%)	Redundancy	
4 λ experiment, 20.0 to 3.0 Å				(3.1–3.0 Å)	
λ_1 0.9871 (pre-edge)	7,519 (4,911)	83.3 (98.0)	0.064	(0.131)	
λ_2 0.9794 (edge)	7,496 (4,891)	83.1 (97.6)	0.067	(0.133)	
λ_3 0.9792 (peak)	7,722 (4,915)	85.6 (98.0)	0.072	(0.149)	
λ_4 0.9686 (remote)	7,475 (4,909)	82.8 (97.9)	0.065	(0.137)	
3 λ experiment, 20.0 to 2.5 Å				(2.6–2.5 Å)	
λ_2 0.9793 (edge)	10,200 (7,820)	68.3 (92.9)	0.060	(0.170)	
λ_3 0.9791 (peak)	10,171 (7,800)	68.1 (92.7)	0.060	(0.169)	
λ_4 0.9686 (remote)	10,140 (7,798)	67.9 (92.7)	0.060	(0.153)	
3 λ experiment, 20.0 to 3.0 Å				(3.2–3.0 Å)	
λ_2 0.9793 (edge)	5,774 (4,635)	66.9 (93.3)	0.053	(0.065)	
λ_3 0.9791 (peak)	5,732 (4,609)	66.4 (92.8)	0.052	(0.066)	
λ_4 0.9686 (remote)	5,710 (4,606)	66.2 (92.7)	0.052	(0.063)	
MAD phasing statistics†					
4 λ experiment, 15.0 to 3.0 Å					
λ_1	λ_2	λ_3	λ_4	3 λ experiment, 15.0 to 2.5 Å	
λ_1 0.061 (0.055)	0.063	0.060	0.054		
λ_2	0.069 (0.058)	0.055	0.065	λ_2 0.068 (0.041)	
λ_3		0.090 (0.054)	0.060	λ_3 0.089 (0.042)	
λ_4			0.075 (0.050)	λ_4 0.072 (0.042)	
MAD phasing‡	$R(\sigma^2 F_T)$	$R(\sigma^2 F_A)$	$\langle \Delta(\Delta\phi) \rangle$	$\langle \sigma(\Delta\phi) \rangle$	$\langle m \rangle$
4 λ experiment					
20.0 to 3.0 Å	0.047	0.413	47.94	21.20	0.86
3.5 to 3.0 Å	0.063	0.441	64.36	26.30	
3 λ experiment					
20.0 to 2.5 Å	0.053	0.391	46.53	22.62	0.88
20.0 to 3.0 Å	0.046	0.371	41.03	20.97	0.89
3.5 to 3.0 Å	0.058	0.431	49.96	23.43	
3.0 to 2.5 Å	0.094	0.469	62.73	27.47	
Refinement statistics§	Resolution range	R-value	$\langle B \text{-value} \rangle$	r.m.s. bonds	r.m.s. angles
	6.0–2.5 Å	20.7%	24.2 Å ²	0.013 Å	1.8 Å

Expression and purification. The sequence coding for *umuD'* was cloned into a pALTER-1 plasmid and the M138T and M138V mutations were made by standard PCR techniques. The gene was sequenced in each case to make sure there were no other mutations introduced by the PCR. The gene was subcloned into a new pALTER plasmid behind the T7 promoter. The protein was then overexpressed in modified DL41 *E. coli* (a DE3 prophage was inserted into the chromosome²⁰) by the addition of 1 mM isopropyl- β -D-thiogalactoside (IPTG) to the growth media. The selenomethionine-substituted protein was produced by growing bacteria in a defined media with selenomethionine¹⁴. The cells were broken by sonication and the cell debris removed by centrifugation. The supernatant was loaded onto a Sepharose Q column and the fractions were screened for UmuD' protein by SDS-PAGE. The samples containing UmuD' were concentrated and were applied to a Superdex 200 sizing column. The fractions containing UmuD' were pooled, concentrated, and loaded onto a Mono Q column. The fractions containing pure UmuD' were then collected, concentrated to 10 to 20 mg ml⁻¹ and stored at -80 °C. **Crystallization.** The protein was crystallized by the hanging drop method in 100 mM cacodylate buffer, pH 5.8, 600 mM Li₂SO₄, 20 mM MgCl₂, 5 mM DTT, 2 g l⁻¹ free D-methionine at 20 °C, with a final protein concentration of 12–15 mg ml⁻¹. Crystals formed over 1–2 weeks and were typically 30 × 30 × 150 μ m in space group P4₁2₁2, with cell dimensions $a = b = 52.8$ Å, $c = 160.1$ Å. There are two UmuD' polypeptide chains per asymmetric unit with a solvent content of 47%. The crystals diffracted to ~3 Å at room temperature on a Rigaku R200 X-ray generator and to beyond 2.5 Å frozen at 100 K in paratone at the synchrotron. **Data collection and processing.** The 4 λ data set was collected at X4A (NSLS) and had usable data to just beyond 3.0 Å. An oscillation range of 3.0° was used with an overlap of 0.5° and exposure times of 240 to 480 s. Bijvoet mates were collected on the same Fuji image plate. The data were processed with DENZO²¹ and scaled using a modified CCP4²² suite of programs, so that redundant reflections were not merged. The MADSYS²³ programs were used to compute the phases and the figures of merit. MULTAN²³ was used to find 3 of the 4 selenium atom sites, and the fourth site was found by difference Fourier techniques. The program ASLSQ¹⁰ was used to refine the selenium site positions. **Model building and refinement.** Maps were calculated in XPLOR²⁴ and displayed with the interactive graphics program O²⁵. The BONES command from MAPMAN²⁶ was used to build a partial backbone into the initial density. The maps were improved by solvent flattening, histogram matching, and non-crystallographic symmetry averaging using the program DM²² once the matrix describing the molecular 2-fold axis was found. These maps were easier to interpret and a model with the UmuD' sequence was built into this density. At this point a second 3 λ data set was collected at beamline X4A under similar conditions to the first data set. These data were substantially better and diffraction went beyond 2.5-Å Bragg spacings. These new data were processed as before, and the model was fitted into these new maps and refined. The model was refined using λ_4 (of the 2.5 Å data set) with the addition of a small number of reflections (less than 10%) from a more complete data set at a remote wavelength that had been scaled to the λ_4 data. The model was refined using simulated annealing, Powell minimization and manual rebuilding using the programs XPLOR and O. NCS restraints were used for all but the initial rounds of refinement, except on residues involved in crystal contacts (residues 32–37, 102, 137). There is little or no side chain density for residues 32, 35, 36 and 42 for both monomers in the molecular dimer. There is little or no density for residues 25–31 and 138–139, although we believe that these residues are still present in the crystallized protein (from mass spectrometry analysis and N-terminal sequencing of the protein). As defined by PROCHECK²⁷, there are no residues in disallowed main-chain torsion angle regions nor in the generously allowed regions. A comparison of the two protomers (A and B) gives an r.m.s. deviation for all NCS restrained atoms of 0.10 Å for backbone atoms and 0.15 Å for side-chain atoms. The r.m.s. deviation on Bs for all backbone atoms is 1.8 Å² and for all side-chain atoms is 2.8 Å². The final R-value for all data having $|F| > 3\sigma = 20.7\%$ and the free R = 30.3%. The final R-value for all data having $|F| > 1\sigma = 21.7\%$.

* Unique reflections and completeness statistics are given considering the Bijvoet mates inequivalent, with the numbers in parentheses considering the Bijvoet mates equivalent.

† Table values represent r.m.s. $\langle \Delta|F| \rangle / \langle |F| \rangle$, where $\Delta|F|$ is the Bijvoet difference at one wavelength (diagonal elements) or the dispersive difference between two wavelengths (off diagonal elements). The values in parentheses are the ratios for centric Bijvoet reflections, which serve as an estimate of the noise in the anomalous data, as these would be zero for perfect data.

‡ $R = \sum_h \sum_i |F_i(h)| - \langle |F(h)| \rangle / \sum_h |F(h)|$. $F_T(h)$ is the structure factor due to normal scattering from all of the atoms, $F_A(h)$ is the structure factor due to normal scattering from the anomalous scatterers only, and $\Delta\phi(h)$ is the phase difference between $F_T(h)$ and $F_A(h)$. $\Delta(\Delta\phi)$ is the difference between independent determinations of $\Delta\phi(h)$. Values given do not include reflections with $m = 0$; $\langle m \rangle$ is the mean figure of merit.

§ A subset of data (6%) was excluded from the refinement and used for the free R calculation.

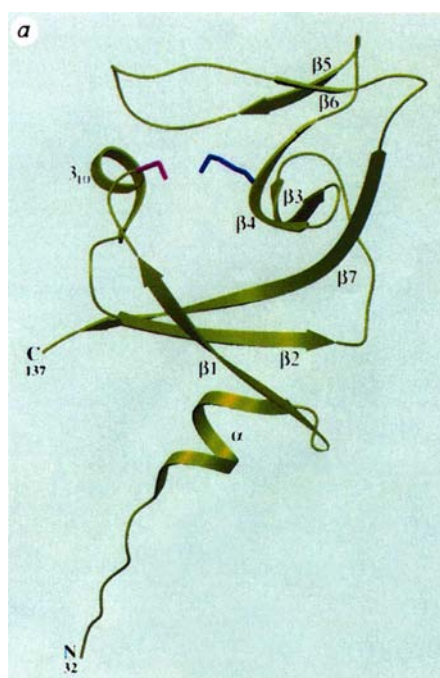
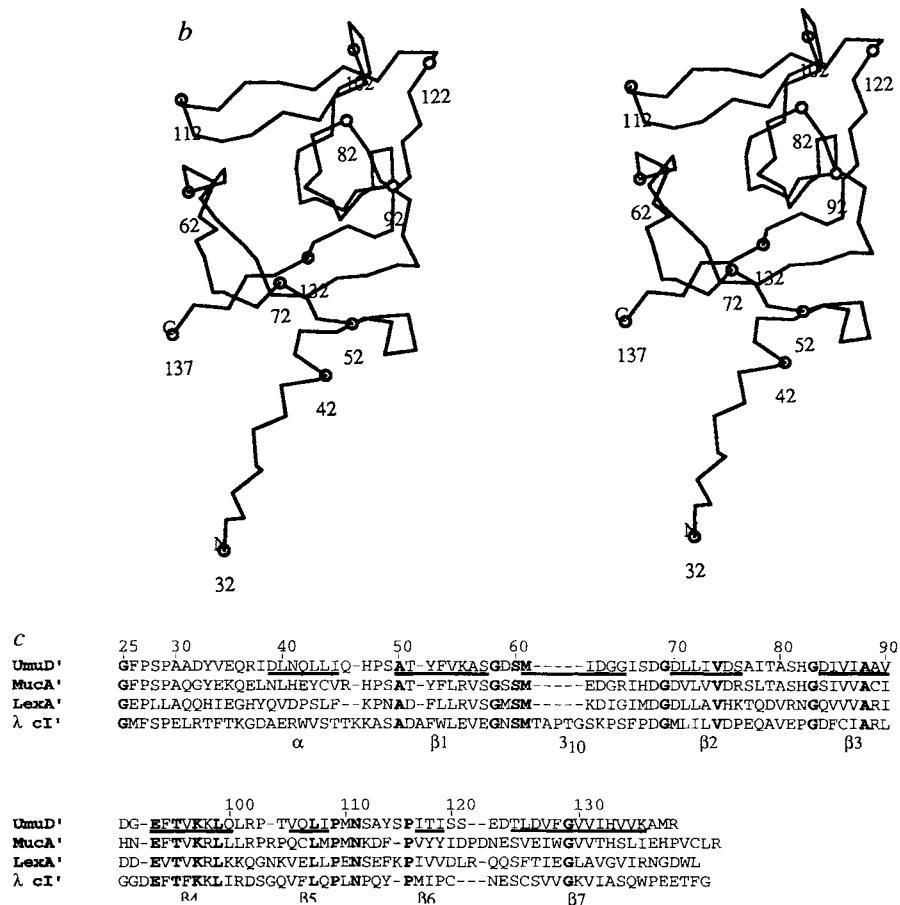


FIG. 1 a, A ribbon diagram of a UmuD' protomer showing the secondary structure elements: the α and 3_{10} helices; and the β_1 - β_7 strands. The active-site residues Ser60 and Lys97 are shown in magenta and dark blue, respectively. Residues 32-137 are shown. b, An α -carbon stereo diagram of the UmuD' protomer in the same orientation as a. Every tenth residue is labelled starting with residue 32. The C-terminal residue, 137, is also labelled. Figures were generated in SETOR²⁸ (a) and MOLSCRIPT²⁹ (b). c, Amino-acid sequence alignment of UmuD' and three homologous proteins, MucA', LexA' and λ cI'. MucA', a plasmid-derived protein with similar function to UmuD', shares a 45% sequence identity with UmuD'. The regulatory dimerization domains of LexA and λ cI are also quite similar, sharing 32% and 26% sequence identity, respectively. Hydrophobic core residues are conserved, and all of the insertions and deletions, except one (a single residue in λ cI), are found in loop regions of the structure. Numbering is according to the UmuD sequence, starting with residue 25,



where the ' refers to the cleaved form of these proteins. All of the sequences start at the self-cleavage site for these proteins. Those residues in bold are conserved among at least 9 of 10 of the λ cI, LexA and UmuD-like proteins (data set consists of λ cI, LexA, *E. coli* UmuD, *S. typhimurium* UmuD, MucA_(R471a), MucA_(R446b), Ruma_(R391), MucA, SamA, ImpA). Those residues underlined are involved in the UmuD' secondary structure labelled below the residues. Secondary structure was determined from PROCHECK²⁷, using the Kabsch and Sanders guidelines. Knowledge of the UmuD' structure was used in the sequence alignment.

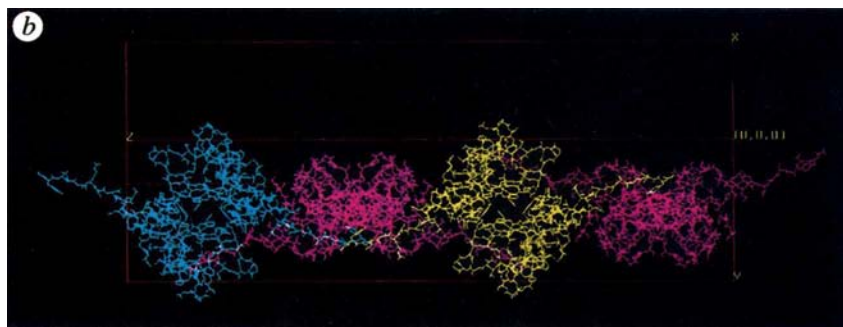
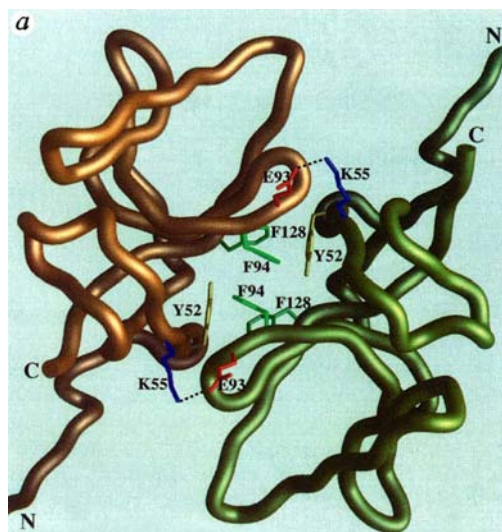


FIG. 2 a, Overall view of dimer interactions, as a worm representation. Hydrophobic side chains are in green, acidic side chains are in red, basic side chains are in blue and all other side chains are in yellow. The dotted line represents a hydrogen bond between the Glu 93 and Lys 55 residues. Note the stacking of the two Phe 94 rings. b, An all-atom, non-hydrogen representation of 4 molecular dimers in the unit cell of the crystal. Each dimer is coloured separately, showing how the filament is formed in the crystal. The molecular diad axis of the yellow dimer is offset by 9.3° from the crystallographic a axis. Figures were made using GRASP³⁰ (a) and O²⁵ (b).

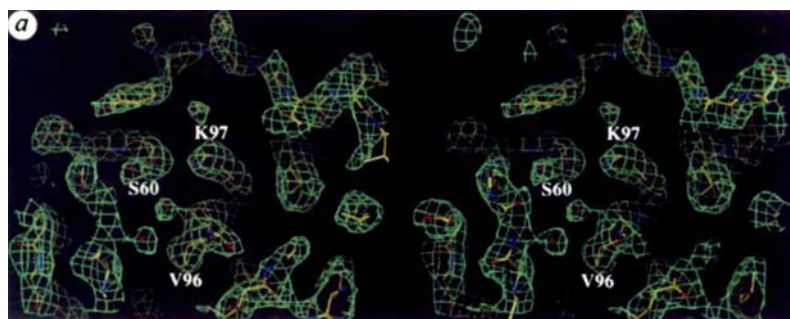
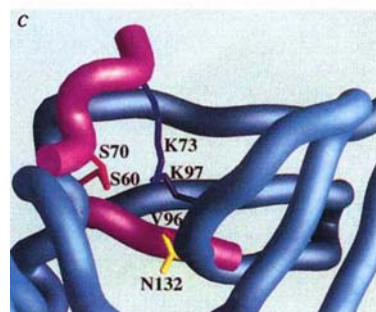
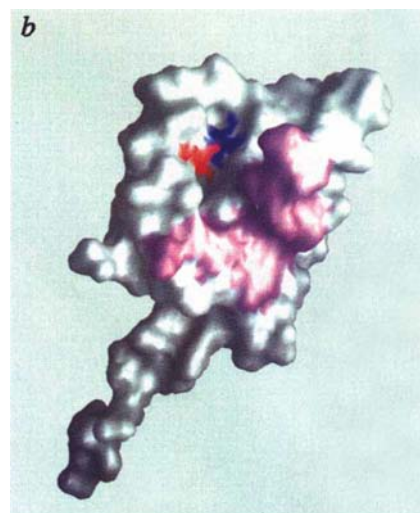


FIG. 3 *a*, Electron density representation of the active site residues Ser60 and Lys97, with a contour level of 1.2σ . A stick model of the structure has been put into the density to show the relevant atoms and bonds. Water molecules are shown as red stars. *b*, A surface representation of the UmuD' protein. The cleft leading to the active-site residues is clear in this representation. The surface contributed by the Ser60 residue is coloured red and that by the Lys97 residue is coloured blue. The residues that contribute to the molecular dimer interface have their surface contributions coloured violet (Tyr52, Val54, Ile87, Gly92, Glu93, Phe94, Phe128). *c*, Worm representation of the UmuD' backbone (yellow) with the sidechains of Ser60 and Lys97 in red and blue, respectively. The backbone carbonyl oxygen of residue Val96 is in red. Superimposed are two segments of the TEM1 β -lactamase structure (in magenta). The first fragment includes residues 69–74 with the Ser70 and the Lys73 side chains in red and blue, respectively. The second fragment includes residues 129–133 with the Asn132 side chain in yellow. An r.m.s. deviation of $0.12\text{ \AA}$ is found when superimposing the Ser60/Ser70 O γ , the Lys97/Lys73 N ϵ , and the Val96 O with Asn132 O δ atoms. In the reaction proposed in ref. 17, Ser70 O γ attacks the carbonyl carbon of the β -lactam ring after activation by proton transfer to Lys73 N ϵ , $2.7\text{ \AA}$ away. The Lys73 N ϵ is positioned by hydrogen bonds to the Asn132 O δ and the Ser130 O γ , holding the amino group in the proper conformation for catalysis. The TEM1 β -lactamase structure coordinates were kindly provided by N. Strynadka. Figures were generated in O 25 (*a*) and GRASP 30 (*b*, *c*).



(manuscript in preparation) and as such provides another example of how physiologically important quaternary interactions are maintained in the crystal lattice^{18,19}. We propose that the UmuD' filament probably forms a scaffold on the RecA–DNA filament that positions UmuC appropriately for interactions with the DNA polymerase III holoenzyme. The mutagenic character of UmuD' necessitates that the protein be tightly regulated, with one level of regulation being the protein's activation by self-cleavage. Formation of the molecular dimer looks to be yet another regulatory mechanism for UmuD, blocking entry of the N terminus to the catalytic cleft, and preventing autodigestion. We are just beginning to understand that the small size of UmuD belies the multitude of interactions this protein has in its role in cell survival. □

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Context-dependent secondary structure formation of a designed protein sequence

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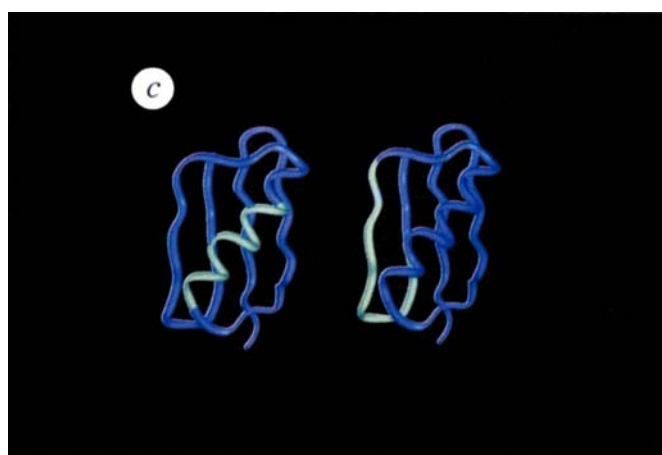
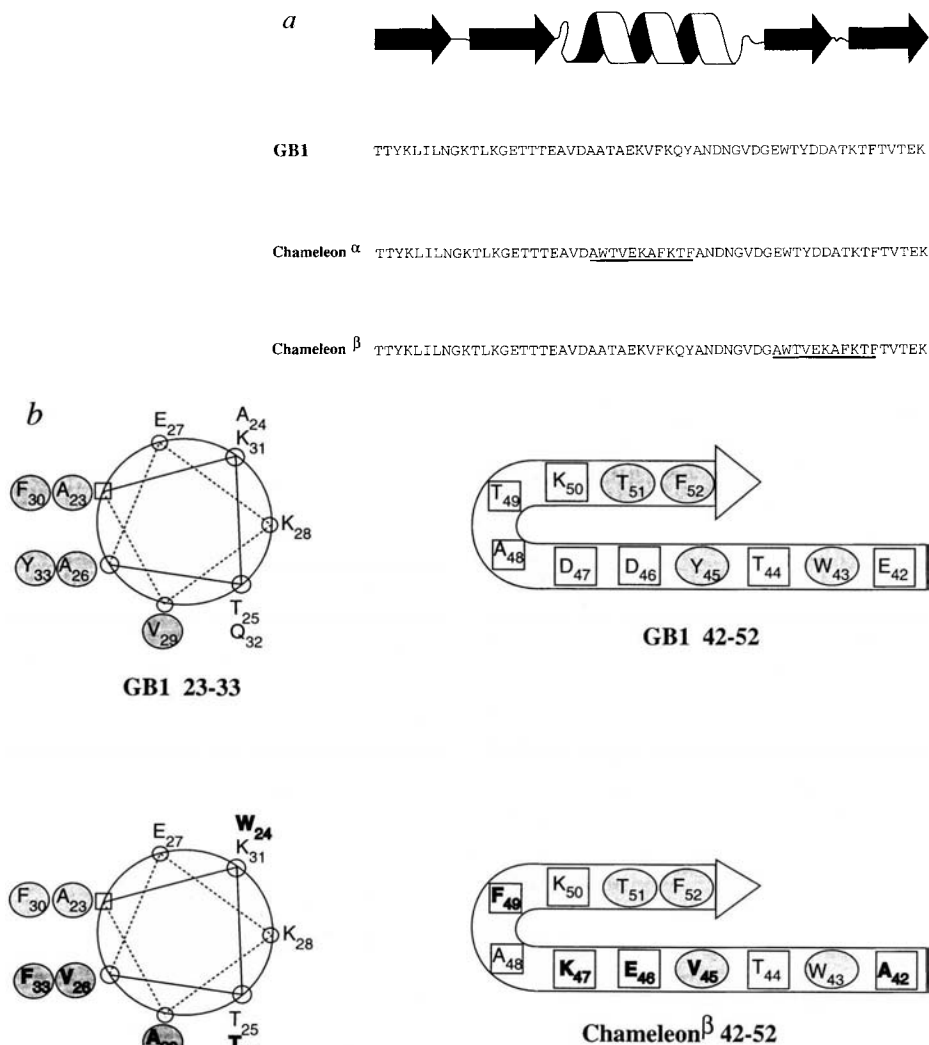
PROTEIN secondary structures have been viewed as fundamental building blocks for protein folding, structure and design. Previous studies indicate that the propensities of individual amino acids to form particular secondary structures are the result of a combination of local conformational preferences^{1,2} and non-local factors^{3–7}. To examine the extent to which non-local factors influence the formation of secondary structural elements, we have designed an 11-amino-acid sequence (dubbed the 'chameleon' sequence) that folds as an α -helix when in one position but as a

β -sheet when in another position of the primary sequence of the IgG-binding domain of protein G (GB1). Both proteins, chameleon- α and chameleon- β , are folded into structures similar to native GB1, as judged by several biophysical criteria. Our results demonstrate that non-local interactions can determine the secondary structure of peptide sequences of substantial length. They also support views of protein folding that favour tertiary interactions as dominant determinants of structure (for example, see refs 8,9).

FIG. 1 a, GB1 secondary structure. The primary amino-acid sequences of GB1, Chm- α and Chm- β are positioned below the corresponding secondary structures. b, Positions 23–33 and 42–52 of GB1 and Chm sequences. Changes from wild-type sequence are indicated in bold. Residues involved in the interface between each secondary structure and the remainder of the protein are shaded. c, Ribbon diagram showing the Chm sequence in Chm- α (left) and Chm- β (right); the Chm sequence is shown in yellow. The diagram was drawn using the coordinates of wild-type GB1 (ref. 21).

METHODS. GB1 mutants were derived from a synthetic GB1 gene by site-directed mutagenesis, verified by dideoxyribonucleotide sequencing, expressed in *E. coli* and purified by IgG-affinity chromatography and reverse-phase HPLC (ref. 22). In the GB1 homologue GB2 (ref. 23), position 57 is incorporated as part of the fourth β -strand. GB1*, with residue K57 added to the 56-residue construct GB1-Thr1 (ref. 22), had a $T_m \sim 2^\circ\text{C}$ higher than GB1-Thr1 and was used as the parent construct for all Chm proteins. Molecular identities were verified by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (Finnigan MAT Lasermat or PerSeptive Biosystems Voyager) and found to be within 2 daltons of the expected mass for the 57-residue protein. The Chameleon sequence positions 23, 26, 30 and 33 were judged to be important for the helix–protein interface, whereas positions 43, 45, 51 and 52 were judged to be important for the β -sheet–protein interface (see text). For all class I sites, the buried residue was used, with the exception of the 29/48 pair. The buried residue of the 29/48 pair was not used as T49F was a necessary change in the turn between strands III and IV of Chm- β to preserve the buried Phe from the 30/49 class I pair, and we did not wish to cause two sequential amino-acid changes in this turn. We tested a number of substitutions for pair 26/45 in GB1*. The T_m (ΔH_m) values from thermal unfolding were: A26Y, $< 0^\circ\text{C}$ (not determined); A26I, 59.6°C ($42.5\text{ kcal mol}^{-1}$); A26V, 66.5°C ($50.9\text{ kcal mol}^{-1}$); Y45A, 53.4°C ($40.4\text{ kcal mol}^{-1}$); Y45V, 61.1°C ($47.5\text{ kcal mol}^{-1}$); Y45I, 61.8°C ($44.5\text{ kcal mol}^{-1}$); thus Val was chosen for the 26–45 pair. Constructs containing a 10-residue Chm sequence at positions 23–32 and 42–51 of GB1* were seen to fold, so the 33/52 pair was examined. Substitution of Y33F lowered the T_m of the α -helix Chm by 0.6°C , whereas F52Y lowered the T_m of the β -sheet Chm by 8.7°C . Phe was chosen for the 33/52 pair in Chm- α and Chm- β .

The 'chameleon' sequence (Chm) was designed to replace α -helix residues 23–33 and β -sheet residues 42–52 (Fig. 1a) of GB1. Our aim was to preserve the hydrophobic nature of the residues that constitute the interface between each of these secondary structure elements and the core of GB1 (Fig. 1b). As the characteristic hydrophobic/hydrophilic patterning of buried residues in α -helices and β -sheets is very different, with periodicities of 3.6 and 2 residues respectively, creation of a sequence consistent with both patterns posed a number of design problems.

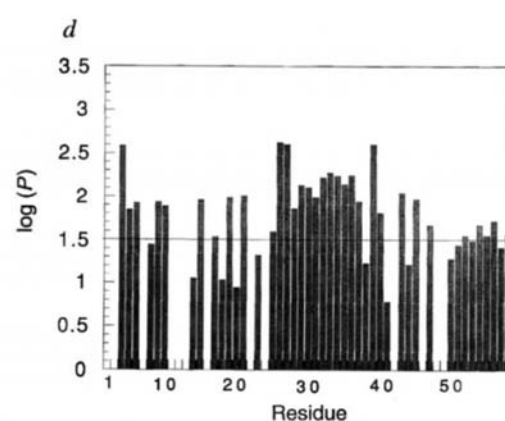
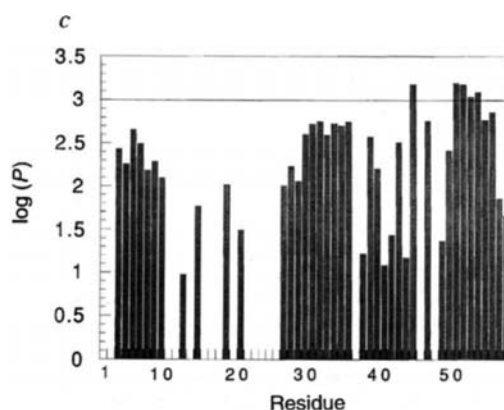
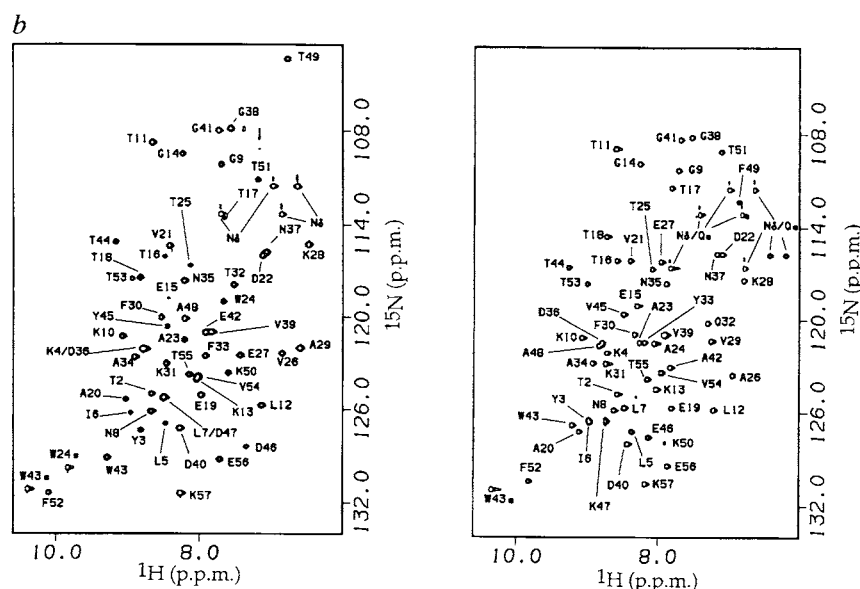
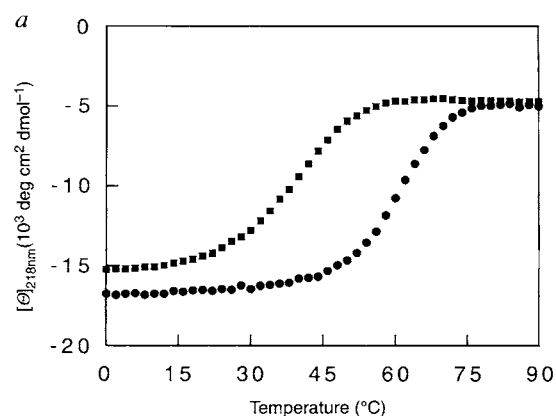


In comparing the structural environments of positions 23–33 and 42–52, we encountered three main types of environments based on accessible surface area: class I, sites where a residue was buried in one secondary structure but exposed in the other (pairs 23/42; 24/43; 29/48; 30/49 and 32/51; the buried residue is underlined); class II, sites where a residue occupied a buried position in

both structures but was very different in size or polarity in each structure (pairs 26/45 and 33/52); and class III, sites that had no conflicts in terms of size, polarity or burial (pairs 25/44; 27/46; 28/47 and 31/50). For class I sites, the buried residue from each pair was used for the Chm sequence, with the exception of pair 29/48 (Fig. 1 legend). For class II sites, a series of hydrophobic

FIG. 2 a, Thermal denaturation of Chm- α (circles) and Chm- β (squares) in 150 mM NaCl, 50 mM sodium acetate, pH 5.4. T_m (ΔH_m) values are: Chm- α , 61.4 °C (41.5 kcal mol⁻¹); Chm- β , 39.2 °C (27.7 kcal mol⁻¹). T_m (ΔH_m) values for wild-type GB1 are 87.5 °C (61.7 kcal mol⁻¹)²⁴. From differential scanning calorimetry (DSC), $\Delta H_{cal} = 44.1$ kcal mol⁻¹ ($\Delta H_{cal}/\Delta H_{van't Hoff} = 1.06$) for Chm- α , consistent with two-state unfolding. DSC of Chm- β gave an unsatisfactory folded baseline at 0–10 °C. b, ¹H–¹⁵N correlation spectra of Chm- α (left) and Chm- β (right) proteins at 5 °C, pH 5.4 (10% D₂O). Labels indicate resonance assignments. The side-chain resonances are labelled as N δ /Q ϵ . At lower contours, the heteronuclear single quantum coherence (HSQC) spectra of Chm- β contain a set of small amide peaks at chemical shifts characteristic of unfolded polypeptides²⁵ which increase in size as the temperature is raised, suggesting that they arise from the unfolded state, in slow exchange with the folded state on the NMR timescale. Measurement of the amide-proton peak volumes indicates that ~1–3% of the molecules are unfolded at 5 °C, as expected from the ΔG of folding for Chm- β . c, Amide-proton protection patterns for Chm- α (c) and Chm- β (d). P is the protection factor from exchange, defined as k_{ex}/k_{obs} , where k_{ex} is the hydrogen-exchange rate expected in a random coil and corrected for primary structure effects²⁶, and k_{obs} is the measured hydrogen-exchange rate. Horizontal lines indicate the log(P) value expected from the global stability of each protein at 5 °C (see also ref. 27).

METHODS. CD thermal unfolding and DSC measurements were made as described²². NMR experiments and resonance assignments were done with ¹⁵N-labelled protein²². For hydrogen exchange experiments, fully protonated Chm- α and Chm- β were adjusted to pH 5.4 and lyophilized from water. Exchange was initiated by dissolution into exchange buffer, 50 mM acetic-d₃-acid-d (Aldrich 99.5 atom % D) in D₂O (Aldrich 100.0 atom % D), pH 5.4 at 5 °C (measured in D₂O using a glass electrode without correction for isotope effects). Proton occupancy was measured from ¹H–¹⁵N HSQC spectra with 2, 4, 16 or 32 transients per increment, with 100 T₁ increments. Amide-proton decay was measured from peak volumes in ¹H–¹⁵N HSQC spectra using FELIX230 (Biosym). Amide-proton-exchange rates were calculated using $I(t) = e^{-(kt)} + I(\infty)$, where $I(t)$ is intensity at time t and k is the measured exchange rate. $I(\infty)$ values were all 0 ± 0.1 . The protection factor P was calculated to account for primary sequence effects²⁶. The global stability of each protein at 5 °C was calculated from CD thermal unfolding measurements and the Gibbs–Helmholtz equation²².



residues were substituted at the positions of the residue pairs in the wild-type GB1 background in order to find a common residue for both environments that did not disrupt protein stability too drastically (Fig. 1 legend). After determining the residues that would enable a single sequence to fulfil the tertiary requirements of both positions 23–33 and 42–52 in GB1 using this procedure, the sequence AWTVEKAFKTF was introduced at positions 23–33 or 42–52 of GB1 by site-directed mutagenesis, thereby creating the proteins Chm- α and - β , respectively (Fig. 1c).

Chameleon- α and - β each display cooperative reversible thermal unfolding as measured by circular dichroism (CD) spectroscopy (Fig. 2a) and differential scanning calorimetry experiments (Fig. 2 legend). This type of thermal unfolding behaviour is a hallmark of compact single-domain globular proteins with uniquely packed hydrophobic cores¹⁰.

The NMR spectra of Chm- α and Chm- β have substantial chemical-shift dispersion (Fig. 2b). In addition, NMR-detected hydrogen-exchange experiments indicate that both proteins contain a set of backbone amides that have exchange rates equal to or slower than those expected for exchange occurring only from global unfolding (Fig. 2c, d). Together with the thermal unfolding data, these results strongly suggest that both Chm proteins have unique folded structures with well packed hydrophobic cores (see discussion in ref. 11).

Nuclear Overhauser (NOE) spectra indicate that, as designed, the Chm sequence is folded into an α -helix in Chm- α (Fig. 3a) and a β -strand/turn/ β -strand in Chm- β (Fig. 3b). In both proteins, amide protons from residues in the Chm sequence are significantly protected from hydrogen exchange, indicating that the hydrogen bonds in each secondary structure formed by the Chm sequence are stable.

NOE patterns throughout each protein are consistent with those in wild-type GB1 (Fig. 4). Furthermore, Chm- α and Chm- β are capable of competing for Fc binding with wild-type GB1, although with somewhat reduced affinities (Fig. 4 legend). The amino-acid changes in Chm- α and Chm- β occur in regions of the protein that have been identified as part of the Fc binding interface¹², so it is likely that much of the affinity differences are the result of mutation of interface residues. Considered together with the NMR data, the fact that Fc binding can occur strongly suggests that both Chm proteins are folded into structures similar to wild-type GB1.

As some short isolated peptides can form significant amounts of secondary structure^{1,13}, an 11-residue peptide corresponding to the Chm sequence (Ac-AWTVEKAFKTF-NH₂, where Ac is acetyl) was synthesized to investigate whether this sequence could have any intrinsic conformational preferences. Both CD and NMR indicate that the Chm peptide is unfolded in isolation (Fig. 4 legend), suggesting that the Chm primary sequence itself has no strong preference for either the α -helix or β -sheet conformation. Thus, the secondary structure formed by the Chm sequence in both Chm- α and Chm- β proteins is specified by tertiary interactions.

The secondary structure of peptide sequences in some natural proteins, such as haemagglutinin and the serpins, undergo major conformational alterations following tertiary rearrangements induced by pH changes or proteolytic cleavage^{14–16}. Tertiary interactions play a dominant role in determining the propensity of individual amino acids to form β -sheets^{3,5,7}; they also affect the secondary structure conformation of identical short peptide sequences (up to 6 amino acids long) in proteins in the structural database^{17–20}. Our Chm sequence design demonstrates directly

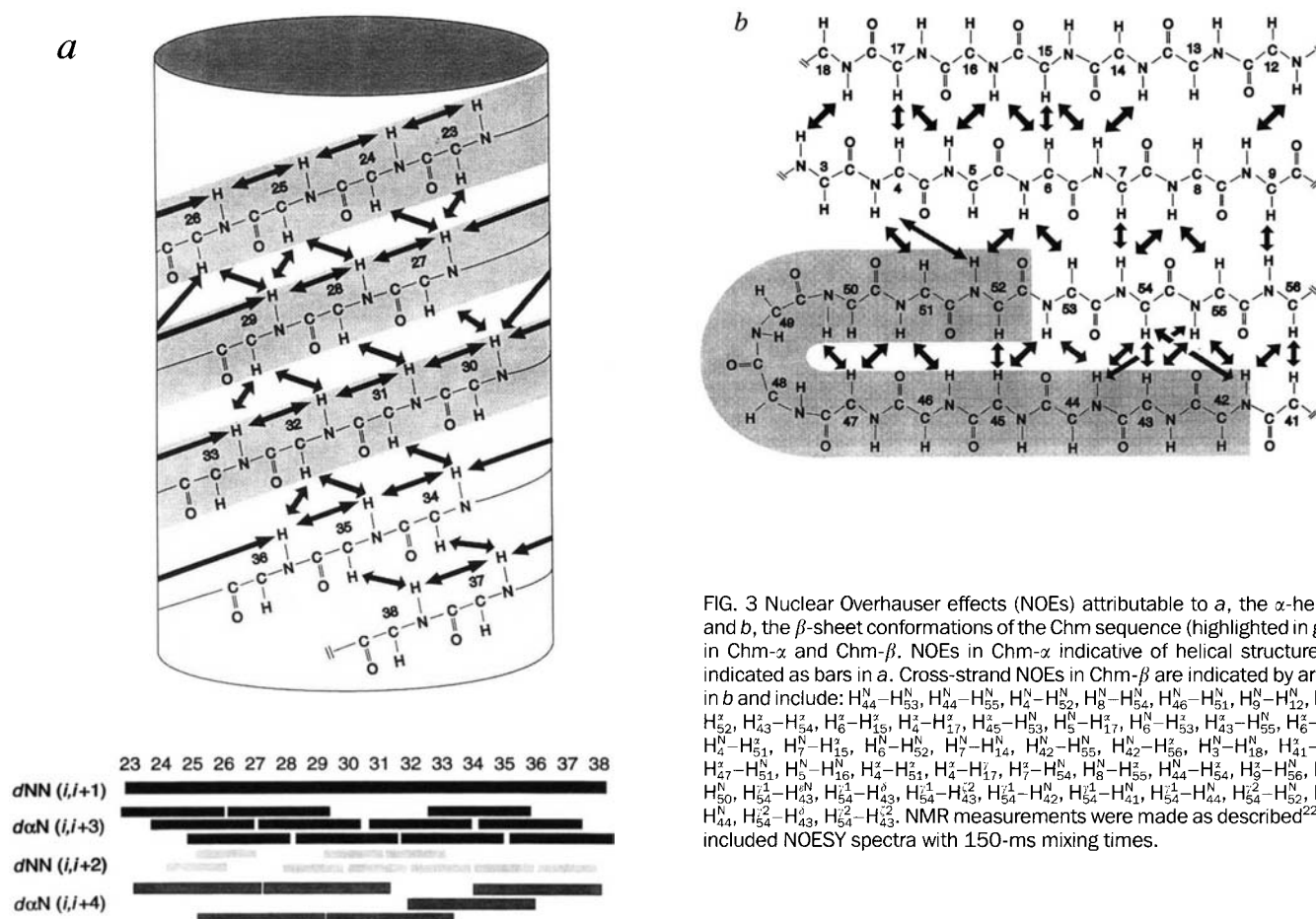
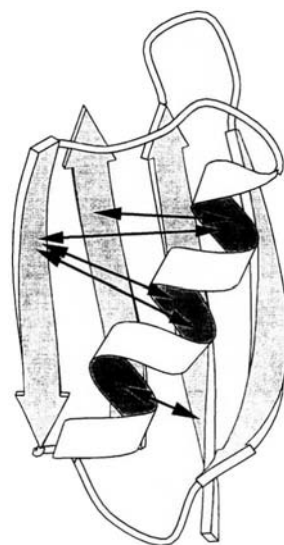


FIG. 3 Nuclear Overhauser effects (NOEs) attributable to a, the α -helical, and b, the β -sheet conformations of the Chm sequence (highlighted in grey) in Chm- α and Chm- β . NOEs in Chm- α indicative of helical structure are indicated as bars in a. Cross-strand NOEs in Chm- β are indicated by arrows in b and include: $H_{14}^N-H_{53}^N$, $H_{44}^N-H_{55}^N$, $H_4^N-H_{52}^N$, $H_8^N-H_{51}^N$, $H_{46}^N-H_{51}^N$, $H_6^N-H_{22}^N$, $H_{45}^N-H_{52}^N$, $H_{43}^N-H_{54}^N$, $H_6^N-H_{15}^N$, $H_4^N-H_{17}^N$, $H_{45}^N-H_{53}^N$, $H_5^N-H_{17}^N$, $H_6^N-H_{53}^N$, $H_{43}^N-H_{55}^N$, $H_6^N-H_{16}^N$, $H_{42}^N-H_{51}^N$, $H_5^N-H_{15}^N$, $H_6^N-H_{52}^N$, $H_7^N-H_{14}^N$, $H_{42}^N-H_{55}^N$, $H_{42}^N-H_{56}^N$, $H_3^N-H_{18}^N$, $H_{41}^N-H_{56}^N$, $H_{47}^N-H_{51}^N$, $H_5^N-H_{16}^N$, $H_4^N-H_{51}^N$, $H_4^N-H_{17}^N$, $H_7^N-H_{54}^N$, $H_8^N-H_{55}^N$, $H_{44}^N-H_{54}^N$, $H_9^N-H_{56}^N$, $H_{47}^N-H_{50}^N$, $H_{54}^N-H_{43}^N$, $H_{54}^N-H_{43}^N$, $H_{54}^N-H_{43}^N$, $H_{54}^N-H_{42}^N$, $H_{54}^N-H_{41}^N$, $H_{54}^N-H_{44}^N$, $H_{54}^N-H_{52}^N$, $H_{54}^N-H_{44}^N$, $H_{54}^N-H_{43}^N$, $H_{54}^N-H_{43}^N$. NMR measurements were made as described²² and included NOESY spectra with 150-ms mixing times.

FIG. 4 Diagram of tertiary NOEs observed between the helix and sheet in both Chm- α and Chm- β . Tertiary NOEs represented by the arrows include: $H_{34}^{\beta} - H_{54}^{\beta}$, $H_{34}^{\beta} - H_{54}^{\gamma 1}$, $H_{34}^{\beta} - H_{54}^{\gamma 2}$, $H_{26}^{\beta} - H_{34}^{\beta}$, $H_{26}^{\beta} - H_{34}^{\gamma 1}$, $H_{26}^{\beta} - H_{34}^{\gamma 2}$, $H_{43}^{\beta} - H_{34}^{\beta}$, $H_{43}^{\beta} - H_{34}^{\gamma 1}$, $H_{43}^{\beta} - H_{34}^{\gamma 2}$, $H_{34}^{\beta} - H_{43}^{\beta}$, $H_{34}^{\beta} - H_{43}^{\gamma 1}$, $H_{34}^{\beta} - H_{43}^{\gamma 2}$, for Chm- α ; $H_{43}^{\alpha} - H_{34}^{\alpha}$, $H_{43}^{\alpha} - H_{34}^{\gamma 1}$, $H_{43}^{\alpha} - H_{34}^{\gamma 2}$, $H_{34}^{\alpha} - H_{43}^{\alpha}$, $H_{34}^{\alpha} - H_{43}^{\gamma 1}$, $H_{34}^{\alpha} - H_{43}^{\gamma 2}$, for Chm- β . Competitive Fc binding experiments with GB1* indicate that both proteins bind Fc. Chm- α and Chm- β have relative dissociation constants (K_d/K_d^{GB1}) for Fc binding of 12.0 and 36.0, respectively; K_d for wild-type GB1 is 7.1 nM²⁸. Binding was assayed at 4 °C as described²². Sedimentation equilibrium give values for relative molecular masses (M_s) of 6,800 (calculated, 6,366) and 6,500 (calculated, 6,246) for Chm- α and Chm- β , respectively with random residuals, indicating that both are monomeric. The observed M_s were independent of protein concentration (10–100 μ M). A peptide, Ac-AWTVEKAFKTF-NH₂, corresponding to the Chm sequence with blocked ends, was examined. Its CD spectrum at 0 °C had features typical of unfolded polypeptides²⁹, was independent of concentration (10–200 μ M) and the temperature dependence of the signal at 222 nm was linear from 0–70 °C (data not shown). NMR spectra showed chemical shift dispersion typical of unstructured peptides²⁵. Chou–Fasman³⁰ values for the Chm sequence ($\langle P_{\alpha} \rangle = 1.15$ and $\langle P_{\beta} \rangle = 1.05$) indicate that this sequence has no strong statistical preference for α -helix or β -sheet formation.

METHODS. Sedimentation equilibrium measurements were made as described⁹ at rotor speeds of 35,000 and 42,000 r.p.m. at 4 °C in 150 mM NaCl, 50 mM sodium acetate, pH 5.4. Dilutions were made into dialysate to obtain 10–100 μ M protein samples. Apparent M_s were calculated by fitting data sets from each sector to a single ideal species model. Partial specific volumes of 0.7323 and 0.7406 ml g⁻¹ were used for Chm- α and Chm- β , respectively, and corrected for temperature³¹. The Chm peptide was synthesized by solid-phase Fmoc methods (Applied Biosystems peptide synthesizer 431A) and purified by Sephadex G-25 size-exclusion chromatography in 5% acetic acid and reverse-phase HPLC purification (on a Vydac C18 column using linear H₂O-acetonitrile gradients and 0.1% trifluoroacetic acid). Chm peptide identity was confirmed by MALDI mass spectrometry, measured as 1,367 (expected, 1,368) daltons. CD spectra of Chm peptide were run in 150 mM NaCl, 50 mM sodium acetate, pH 5.4; NMR spectra were obtained in 10% D₂O, pH 5.4.



that the information specifying α -helix or β -sheet secondary structures can be entirely non-local. Taken together, these results underscore the importance of context-dependent effects in protein folding. □

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RETRACTION

Long-term correction of rat model of Parkinson's disease by gene therapy

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Nature **362**, 450–453 (1993)

J.A.W. writes — In my laboratory we have been attempting to extend the findings reported in this paper. During these efforts, it has come to my attention that the pertinent laboratory notebooks were replaced with edited text and data. An independent analysis of the remaining original data revealed that the published Fig. 2b and c contains errors that exaggerate both the reductions in the number of rotations after transplantation and the increments in the numbers of rotations following graft removal. Review of the protocol reported in the legend to Fig. 2 indicates that control transfections were done using TransfectACE (Promega) instead of Lipofectin (BRL), which may have affected the outcome of the experiments. Subsequent experiments have failed to replicate the original observations. Regrettably, therefore, I am unable to verify that the conclusions of this paper are correct. □